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Industrial Chemical Processes

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Polyhydroxyalkanoates production in aerobic sequential processes by mixed microbial cultures

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CHAPTER 1. INTRODUCTION

The current emphasis on sustainability, eco-efficiency and green chemistry has led to intensive search for renewable and environmentally friendly resources. Thus, sustainable development is recognized to be essential for the growth of the economy and industrial productivity. Since global petroleum reserves are finite, there is a need for additional new sources of durable materials. Renewable materials from microorganisms can provide a source of sustainable alternative to petroleum derived chemicals including polymers. integrating renewable feedstocks into the economy could lower crude oil demand, thus limiting economic downturns in the chemical industry due to oil price volatility (Bozell and Landucco 1993; Akaraonye et al. 2010). It would also expand the options of the chemical industry by increasing feedstock flexibility and broadening the spectrum of potential chemical products. Hence, this would provide an acceptable answer to the current problemswith petroleum-based chemicals.

The production of petroleum-based plastics results in the generation of toxic and recalcitrant waste whereas the production of biodegradable polymers involves aqueous processing environments during microbial cultivation, generation of non-toxic waste and the use of renewable non-fossil feedstocks. Biodegradable polymers release carbon dioxide and water vapour into the air while undergoing biological decomposition and growing biomass during photosynthesis subsequently absorb the released carbon dioxide. Hence, the problems associated with conventional petroleum-based plastics have brought biodegradable polymers to the forefront.

Currently, different types of biodegradable polymers are being studied for different applications ranging from the production of everyday used products to medical applications. Numerous biodegradable polymers are being studied including polyhydroxyalkanoates (PHA), polylactide (PLA), poly(ε-caprolactone) (PCL), poly(p-dioxanone) (PPDO) and poly(butylene succinate) (PBS).

PHA are one of the relatively newer families of biodegradable polymers that have great potential in the future due to their variability in properties, they are polyesters of hydroxyalkanoic acids that have received extensive attention due to their inherent biocompatibility and biodegradability.

Microorganisms in nature are able to degrade PHA using PHA depolymerises; however, the activities of these enzymes vary depending on the composition, crystallinity, additives and the surface area of the polymer. In addition, environmental conditions including temperature, moisture level and pH affect the rate of degradation. The end product of PHA degradation in an aerobic environment is carbon dioxide and water while in anaerobic conditions it is methane (Ojumu et al. 2004). In the context of medical applications, PHA are established as biocompatible and biodegradable polymers. A detailed description of the biocompatibility of PHA is reviewed in Valappil et al. (2006).

The most widely applied strategy for PHA production is to cultivate one wild-type or genetically recombinant bacterial strain with a renewable but refined substrate. This has lead to production costs that have been significantly higher than for conventional plastics. Although several commercial PHA products are available such as Biopol™, Biomer™, Biocycle™ and Nodax™ (Lemos et al. 2006), widespread application of PHA is still very limited.

At the same time, some of the bacteria naturally occurring in open mixed microbial cultures (activated sludge) treating wastewaters are able to store PHA. In the wastewater treatment plant, carbon storage in the form of PHA can be enhanced under dynamic conditions respect to microbial substrate. In this way, while the wastewater treatment is applied favouring the PHA storing bacteria, a biopolymer can be harvested as a value-added resource from wastewater treatment system. Treatment of industrial wastewaters exhibiting high organic content and low nitrogen and phosphorous concentration should be especially suitable to couple to PHA production. From the treatment perspective, this will simultaneously give benefits such as reduced amounts of waste sludge as well as reduced energy and nutrient requirements.

CHAPTER 2. PROPERTIES AND BIOSYNTHESIS OF POLYHYDROXYALKANOATES

2.1 Polyhydroxyalkanoates (PHA): structure, properties and application

Global demand of plastic-based materials has increased considerably over the last years (Chanprateep, 2010). However, synthetic polymers that dominate plastics are derived from limited fossil resources and represent a serious hazard to the environment due to their high recalcitrance to biological degradation. The concern associated with the disposal of synthetic polymers has prompted much interest in the research of alternative biodegradable materials, such as polysaccharides, polylactides, and polyhydroxyalkanoates (PHA); the latter being considered among the most promising candidates.

PHA are biologically synthesized polyesters (figure 2.1) that can be produced from renewable resources and are natural, recyclable, biocompatible, and completely biodegradable to water and carbon dioxide (Jendrossek and Handrick, 2002). These biopolymers can exhibit a broad range of thermoplastic and elastomeric properties depending on the incorporated monomers. Over 150 different types of hydroxyalkanoate (HA) monomers have been identified and there is an enormous variation possible in the length and composition of the side chains. In general, PHA monomers can be classified into two main groups: (i) short chain length (SCL) monomers with 3-5 atoms of carbon and (ii) medium chain length (MCL) monomers which consist of a number of carbon atoms between 6 and 14. The average number of repeating units in PHA chain is up to around 30000. An example of SCL-PHA is the poly(3-hydroxybutyrate) [P(3HB)] that, by far, is the most widely studied polyhydroxyalkanoate. P(3HB) is a highly crystalline (55-80%), stiff, but brittle material. The elongation to break (flexibility) is about 2-10% for P(3HB), compared to 400% for polypropylene. Other mechanical properties, such as Young's modulus (3.5 GPa) and tensile strength (40 MPa) are similar to those of polypropylene (Lee, 1996a). The melting temperature of P(3HB) is close to 180 °C and the glass transition temperature is around 4 °C; but the polymer starts to be thermally degraded at 170 °C (Kunioka and Doi, 1990). This means that a reduction in

molecular weight that deteriorates mechanical properties may occur during processing of pure P(3HB) in melt (Carrasco et al. 2006).

Copolymers are obtained by incorporation of other types of monomers than 3HB such as 3HV or 3HHx. This reduces the cristallinity by disturbing the crystal lattice. The copolymers have both increased flexibility (higher elongation to break) and lower melting temperatures than P(3HB). The temperature at which degradation and decomposition of PHA occurs is rather insensitive to the monomer composition. Therefore, the lower melting temperatures of copolymers enables processing with reduced molecular weight losses. The longer the side chain of the monomers, the larger is the disturbing effect on the crystal lattice. This means that in copolymerization with 3HB, MCL monomers such as 3HHx have higher impact on the polymers properties than, for instance, 3HV (Doi et al. 1995).

The microstructures of P(3HB-3HV) copolymers are generally such that the monomers are distributed statistically randomly (Dai et al. 2008; Kamiya et al. 1989). Due to the so called isodimorphism of P(3HB-co-3HV), the melting temperature decreases from 0 to around 30 mol-% 3HV but increases again at higher 3HV content (Bluhm et al. 1986; Feng et al. 2002; Kamiya et al. 1989). Cocrystallization of the two monomer units in either of the homopolymer crystal lattices of P(3HB) or P(3HV) occurs depending on the monomer fractions.

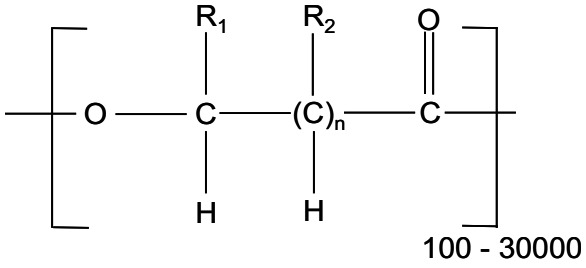


Figure 2.1. PHA general structure: n is the number of carbon atoms in the linear polyester structure, R_1 and R_2 are variable hydrocarbon side chains

The enormous possible variations in the length and composition of the side chains make the PHA polymer family suitable for an array of potential applications ranging from packaging, compost bags, agricultural films (e.g., mulch films), manufacturing disposable everyday articles (e.g., shampoo bottles and cosmetic materials), bulk

commodity plastics, to medical uses (Bucci et al. 2005). Indeed, recent studies have demonstrated the use of PHA in the production of stents and in the tissue engineering of heart valves (Bunger et al. 2007).

Biological degradation of PHA in the environment depends on many factors both related to the environmental conditions (temperature, moisture level and pH) and to the polymer (composition, cristallinity and surface area). Degradation occurs at the surface by enzymatic hydrolysis. These extracellular PHA-degrading enzymes are excreted by many bacteria and fungi in soil and seawater for the purpose of utilizing oligomers and monomers as nutrient. P(3HB-3HV) with 1 mm thickness was completely degraded after 6, 75 and 350 weeks in anaerobic sewage, soil and sea water, respectively (Lee, 1996a)

PHA are synthesized by many types of microorganisms, over 300 species, including both gram-negative and gram-positive bacteria (e.g., *Lampropaedia, Pseudomonas*, *Rhodobacter, Ralstonia*, *Bacillus, Clostridium*), aerobic photosynthetic bacteria (cyanobacteria), anaerobic photosynthetic bacteria (non-sulphur and sulphur purple bacteria), and certain Archea. They function as carbon and energy storage compounds and are present in the cell cytoplasm as insoluble granules (with diameters from 0.2 to 0.7 μm, figure 2.2) (Anderson and Dawes, 1990; Braunegg et al. 1998).

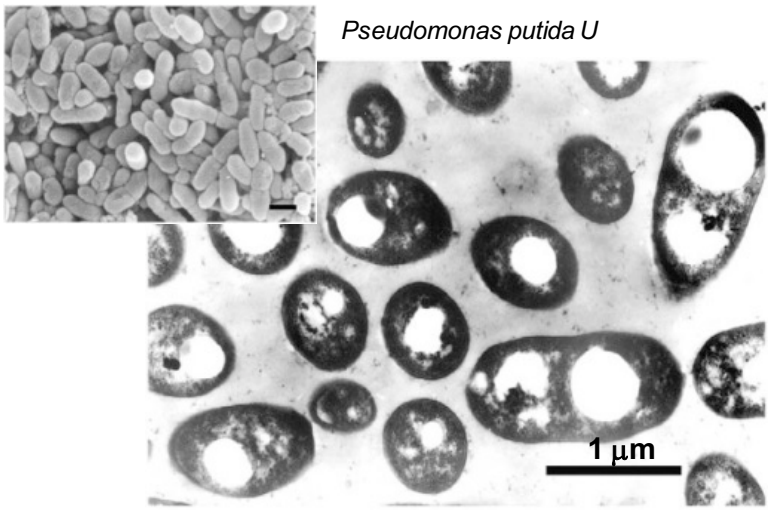


Figure 2.2. PHA granules inside *Pseudomonas putida.*. Current Opinion in Microbiology, 6:251-260 Luengo et al. 2003

The PHA storage occurs when microorganisms are unable to grow at the same rate as they are able to take up the substrate. The restriction in growth rate can be due to limited availability of essential growth nutrients (such as nitrogen, phosphorus, sulphur or oxygen) or to limitations in anabolic enzyme levels and activity.

2.2 PHA production processes employing pure microbial cultures

Presently, PHA production at industrial scale is based on pure culture fermentations. The most commonly used microorganisms are *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Pseudomonas oleovorans*, *Protomonas extorquens*, *Alcaligenes latus*, or recombinant *Escherichia coli* (Chen, 2010; Shi et al. 1997). The culture conditions required for polymer storage are different for the different microorganisms. Most of PHA-producing bacteria require the limitation of an essential nutrient and a two-stage fed-batch process is typically employed. The culture is first supplied with a medium rich in nutrients and carbon sources and, when a high cellular density is reached, growth limiting conditions are imposed in order to induce PHA synthesis. During this limited growth stage an efficient polymer production occurs, either when the carbon source is provided under nutrient-deficient conditions (such as in *P. oleovorans* and *P. extorquens*) or when the nutrient (e.g., nitrogen source) is completely depleted in the medium (such as in *C. necator*) (Suzuki et al. 1986). A more restricted group of bacteria, including *A. latus* and recombinant *E. coli* containing the PHA biosynthetic genes, does not require nutrient limitation and can accumulate PHA during active growth (Page and Cornish, 1993). For the fed-batch culture of these microorganisms the development of a nutrient feeding strategy is crucial to the success of the fermentation, since cell growth and PHA accumulation need to be balanced in order to avoid incomplete polymer accumulation. However, nutrient limitation can further enhance PHA storage also in *A. latus* (Wang and Lee, 1997). Overall, even though maximum PHA contents of up to 80-90% of the cell dry weight have been reported (Groete et al. 1999; Steinbüchel and Lütke-Eversloh, 2003), well-defined substrates and aseptic process conditions are required.

This typically results in high substrate costs, expensive equipments, and high energy consumption, making industrial biotechnological processes based on pure cultures

unfavorable for further exploitation in PHA production. In particular, the substrate cost accounts for about 50% of the total PHA production costs, to which the carbon source contributes most significantly (Kim, 2000), because substrates most commonly used in the industrial processes are pure sugars, such as glucose or sucrose, or other sugar based compounds, such as corn, which have a high market price (Reddy et al. 2003).

C. necator and *A. latus* both use the Entner-Doudoroff patway for carbohydrate catabolism resulting in pyruvate and generation of energy (adenosine triphosphate, ATP) and reducing equivalents (nicotineamide, adenine dinucleotide, NADH). The pyruvate can be converted to acetyl coenzyme A (acetyl-CoA) which enters the tricarboxylic acid (TCA) cycle. In the TCA cycle, terminal oxidation into CO_2 occurs alongside generation of anabolic precursors and additional energy (guanosine triphosphate, GTP) and reducing equivalents (NADH; nicotineamide adenine dinucleotide phosphate, NADPH; and flavin adenine dinucleotide, $FADH_2$). With oxygen as terminal electron acceptor, reducing equivalents are consumed for generating ATP during oxidative phosphorylation.

Instead of being oxidized to CO_2, acetyl-CoA can be converted into P(3HB) (figure 2.3).

If protein synthesis ceases, for instance due to the lack of nitrogen, the cells will experience higher concentration of NADH and NADPH. These inhibit citrate synthase and isocitrate dehydrogenase which will cause the TCA cycle to slow down and direct acetyl-CoA to P(3HB) synthesis (Dawes and Senior 1973). Biosynthesis of P(3HB) from acetyl-CoA in *C. necator* is dependent on three enzymes (Oeding and Schlegel 1973). First two units of acetyl-CoA are joined into acetoacetyl-CoA by 3-ketothiolase (PhaA). Acetoacetyl-CoA is reduced by the NADPH dependent acetoacetyl-CoA reductase (PhaA) into (*R*)-3-hydroxybutyryl-CoA which is subsequently incorporated into the polymer chain by PHA synthase (PhaC). Acetate, propionate, butyrate and valerate are all converted into their respective acyl-CoA. Propionyl-CoA can be combined with acetyl-CoA into 3-ketovaleryl-CoA by 3-ketothiolase and reduced and incorporated in the polymer as 3HV. Propionyl-CoA can also undergo decarboxylation to acetyl-CoA. This is usually the case when propionate is the sole carbon source in order to produce acetyl-CoA for the TCA

cycle and for 3HB and 3HV synthesis. Butyryl-CoA and valeryl-CoA undergo beta oxidation and are thereafter converted into 3HB and 3HV, respectively.

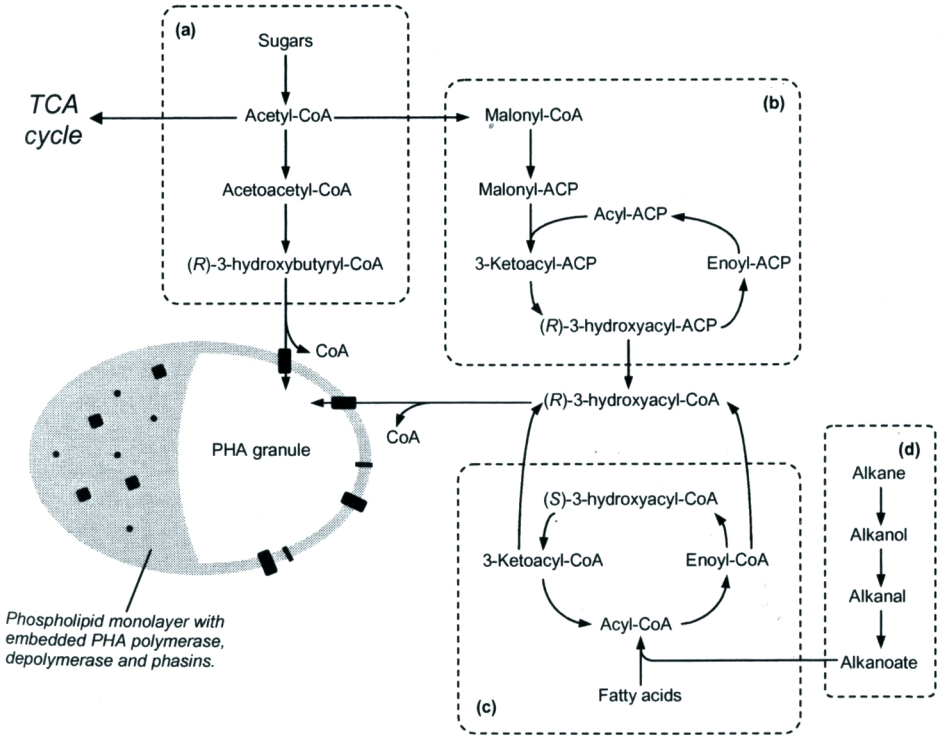


Figure 2.3. Overview of different pathways involved in PHA synthesis. PHA biosynthesis from carbohydrates (a), fatty acid biosynthesis (b), fatty acid β-oxidation (c), and alkane oxidation (d). (Luengo et al. 2003).

Precursor for the PHA synthesis can also be provided through alkane oxidation, fatty-acid β-oxidation and fatty acid synthesis. Monomers of MCL type can be supplied either through β-oxidation of fatty acid (Chen et al. 2001) or from structural non-related substrates such as sugars through fatty acids synthesis (Huijberts et al. 1992; Kato et al. 1996; Nomura et al. 2005). The MCL monomers produced in fatty acid synthesis are transferred from the acyl carrier protein (ACP) to CoA by transacylase protein, PhaG (Rehm et al. 1998).

The carbon and energy stored can be recovered by consuming the PHA when required by the microbial cells. Degradation of P(3HB) in *C. necator* is initiated by PHA depolymerase (PhaZ) and the product of its hydrolysis, (*R*)-3-hydroxybutyrate, is further converted into acetyl-CoA for catabolic and anabolic purposes. In addition

to the catabolic proteins mentioned, non-catabolic proteins, phasins (PhaP), are also associated with the granules. The phasins have hydrophobic domains that allows them to attach to the granules and they are likely involved in the formation and stabilization of the granules (Potter and Steinbuchel 2005; York et al. 2001).

The three enzymes involved in P(3HB) synthesis (PhaA, PhaB, PhaC) are encoded by the single gene cluster *phaCBA* which is subjected to transcriptional control (Slater et al. 1988). Cloning and expression of the *phaCBA* cluster in *Escherichia coli* has been widely explored with purpose of establishing metabolic regulatory mechanisms as well as developing improved strategies (Lee et al. 1996; Lee and Chang 1995; Lee et al. 1999; Slater et al. 1988).

Many variants of PHA synthase exists and these are classified into three types depending on their substrate specifities and primary structures (Rehm and Steinbuchel 1999). The first type is represented by the PHA synthase of *C.necator* and incorporates mainly SCL monomers of 3-, 4- and 5-HA. Some PHA synthases in this group also incorporate MCL monomers such as 3-hydroxyhexanoate, but usually to a very small extent compared to the SCL monomers (Sudesh et al. 2000). The second type of PHA syntheses are able to efficiently incorporate MCL monomers and are, for instance, represented by the two PHA synthase of *Pseudomonas oleovorans*. The third types of PHA synthase are characterized by the fact that they consist of two subunits as for instance in *Chromatium vinosum*. Generally, these prefer SCL monomers but some of them also incorporate both SCL and MCL monomers (Sudesh et al. 2000).

Much effort has been devoted to the development of pure culture fermentation strategies for economical production of PHA. The focus has been to maximize cellular PHA content, cell growth rate and polymer production rate. Strategies that have been applied include the use of wild-type producer organism such as *C. necator* and *A. latus* with different nutrient limitations and cloning and expression of the *C. necator* biosynthetic genes in other organisms such as *E. coli* (Lee et al. 1999). The cloning approach has potential advantages in higher growth rates and broader substrate specificities that could include wastes. In the case of P(3HB), up to 88% polymer of cell dry weight has been obtained using *A. latus* (Wang and Lee, 1997). Even though several brands of PHA are commercially available, the production costs

have been too high for widespread application of these products (Khanna and Srivastava 2005).

2.3 PHA production processes employing mixed microbial cultures

In recent years, there has been considerable interest in investigating alternative low-cost processes for PHA production and much research efforts are particularly being dedicated at exploring the use of low value substrates and mixed microbial cultures (MMCs) (Dionisi et al. 2004; Dionisi et al. 2006; Chua et al. 2003; Salehizadeh et al. 2004; Reis et al. 2003; Beccari et al. 2002; Dircks et al. 2001). In this case, the storage is induced in the microorganisms through a different mechanism with respect to pure cultures; for example it can be induced by internal limitations to growth, intermittent substrate feeding, oxygen limitation (Majone et al. 1999; Beun et al. 2000; Beun et al. 2002).

The combined use of waste feedstocks and MMCs is expected to decrease both investment and fermentation operating costs by simplifying equipments and saving energy (as sterile conditions are not required), as well as reducing the cost of the substrate. Indeed, mixed culture PHA production processes allow using complex substrates of undefined composition possibly contained in agro-industrial waste effluents, thereby coupling the biopolymer production to wastewater treatment. In recent years, a lot of research has been dedicated on the production of biodegradable polymers (e.g. polyhydroxyalkanoates, PHA) from renewable resources: fermented sugar cane molasses (Albuquerque et al. 2007; Albuquerque et al. 2010), fermented palm oil mill effluents (POME) (Din et al. 2006; Salmiati Ujang et al. 2007), fermented paper mill wastewater (Bengtsson et al. 2008), tomato cannery waste (Liu et al. 2008), fermented brewery wastewater (Mato et al. 2008), olive oil mill effluents (Beccari et al. 2009; Dionisi et al. 2005a; Ntaikou et al. 2009).

MMCs are microbial populations operating in open biological systems, whose composition depends directly on the substrate mixture and operational conditions imposed on the open biological system. These have been so far mainly used in biological wastewater treatment plants. However, the ecological role of internal storage products such as PHA has been recognized as an important aspect of activated sludge systems submitted to dynamic conditions, mainly caused by

discontinuous feeding and variations in redox conditions (electron acceptor presence or absence) (Beccari et al. 2002; Satoh et al. 1999).

PHA synthesis by mixed cultures was first observed in wastewater treatment plants designed for biological phosphorous removal (EBPR), which are operated with alternating anaerobic/aerobic cycles. The main groups of bacteria responsible for PHA accumulation in EBPR systems are polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs). Under anaerobic conditions, PAOs and GAOs take up the carbon substrate and store it as PHA, while glycogen, a second storage polymer, is consumed. PHA are used in the successive aerobic phase for cell growth, maintenance, and glycogen pool replenishment. Though the carbon metabolism of PAOs and GAOs is similar, the main difference between them is related to the cycling of polyphosphate by PAOs. Under anaerobic conditions, PAOs release phosphate gaining energy for PHA accumulation, while in the presence of oxygen phosphate is taken up in excess for polyphosphate pool replenishment, and PHA are degraded for storing energy (Pereira et al. 1996). Under anaerobic conditions, on the contrary, GAOs derive energy only by glycogen catabolism through glycolysis and no phosphate is released to the medium or accumulated inside the cells (but the one required for growth).

2.3.1 Feast and famine condition as selective pressure on the biomass

Storage phenomena are induced by dynamic conditions with respect to carbon substrate, which have been referred to as “feast and famine” conditions (Chiesa et al. 1985). These conditions have been widely applied as a strategy to prevent separation problems that are caused by filamentous bulking of activated sludge (Chiesa and Irvine 1985; Daigger and Grady 1982). Since the feast and famine conditions promote a storage response and therefore impose a selective pressure on the microbial community that favors organisms with storage ability, feast/famine regime has been suggest as a strategy for production of PHA from waste or wastewater by open mixed cultures (Majone et al. 1996; Majone et al. 1999; Satoh et al. 1998; Satoh et al. 1999).

Significant PHA storage ability was also observed in activated sludge operating in aerobic wastewater treatment plants where selectors for bulking control were

introduced (Majone et al. 1996). This process configuration is characterized by alternating periods of excess and lack of external carbon substrates, which create internal kinetic limitations to growth and enhance PHA accumulation.

Carbon substrate can be converted into storage polymers such as PHA or into active biomass. In presence of oxygen, a portion of the substrate will also be oxidized to carbon dioxide through respiration in order to produce the energy (ATP) and reduction (NADPH) equivalents necessary to drive the processes and for cellular maintenance. Following a certain period in the absence of external carbon substrate, a decrease of the amount of intracellular components (RNA and enzymes) required for cell growth occurs. When carbon substrate becomes available again, the amount of enzymes present in the cells may not be enough to ensure that a maximum growth rate is reached (van Loosdrecht and Heijnen 2002; van Loosdrecht et al. 1997) and, since fewer enzymes are required for PHA storage, the latter phenomena can occur at a faster rate than cell growth and becomes dominant (Daigger and Grady, 1982; Frigon et al. 2006). These concept are schematically depicted in figure 2.4.

Thus, the restriction of the growth rate can be due to external limitations such as absence of essential growth nutrients (nitrogen, phosphorous, sulphate etc...) or internal limitation such as insufficient amounts and activity of cellular components (RNA and enzymes) required for growth. Internal limitation are believed to be the most important factors for enrichment under feast and famine conditions (Daigger and Grady 1982) and indeed, during a typical feast phase storage occurs with high yields (0.6 C-mol PHB/C-mol acetate) even though sufficient growth nutrients are available (Beun et al. 2002; Dionisi et al. 2004).

An effective selective pressure to enrich activated sludge in PHA producing microorganisms is the application of a cycling feast and famine regime, that is the repeatedly alternating presence (feast phase) and absence (famine phase) of excess substrate (van Loosdrecht et al. 1999; Dias et al. 2006; Wilderer et al. 2001; Morgan-Sagastume et al. 2010). In this way, a competitive advantage exists for microorganisms that are quicker to store the substrate during the feast phase and reuse it as an internal carbon and energy source for cell growth and maintenance during the famine phase. This is how, a culture exposed to alternating feast and famine conditions can be selectively enriched in organisms with PHA storing ability.

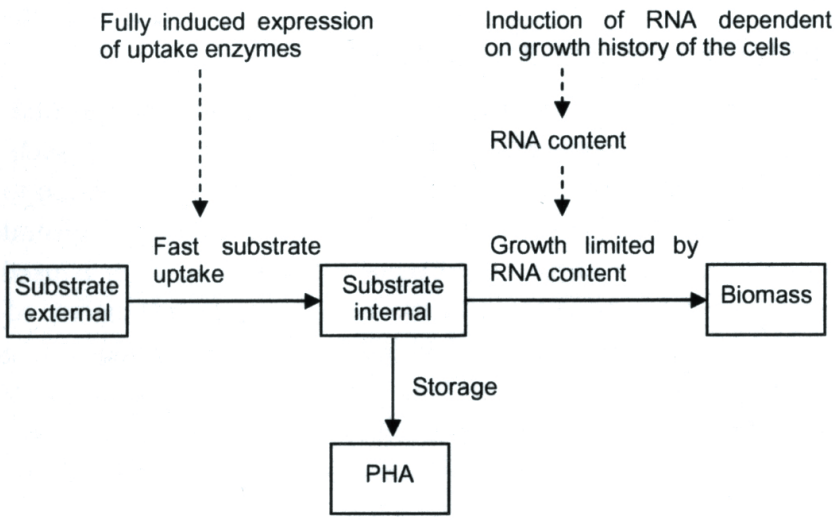


Figure 2.4. Schematic representation of the substrate flux in a microbial cell and the factors which are influencing the metabolic pathway (Krishna and van Loosdrecht 1999).

Under feast condition, substrate is primarily used for PHA storage and under famine conditions, the stored polymer is used for microbial growth and maintenance (figure 2.5). Due to the high energetic efficiency of the storage metabolism, the biomass yield is only around 6% lower with intermediate storage in form of PHB, compared to direct growth on acetate (van Aalst-van Leeuwen et al. 1997). Nevertheless, the slight lower biomass yield with intermediate storage means that enrichment of a storage response is controlled by kinetic rather than thermodynamic factors.

In general, the definition of feast and famine regime can be referred to both (i) the fully aerobic dynamic feeding (ADF) and (ii) the dynamic conditions of oxygen availability (anaerobic/aerobic cycles). The imposition of anaerobic/aerobic cycles is triggering for storage only if the substrate is available in the absence of the final electron acceptor limiting cell growth, whereas the presence of substrate throughout the full cycle would not enhance PHA storage. On the other hand, in anaerobic/aerobic feast and famine regime cell growth occurs only from the intracellular stored polymer, whereas under fully aerobic conditions cell growth can also occur from the external substrate simultaneously to PHA accumulation during the feast phase.

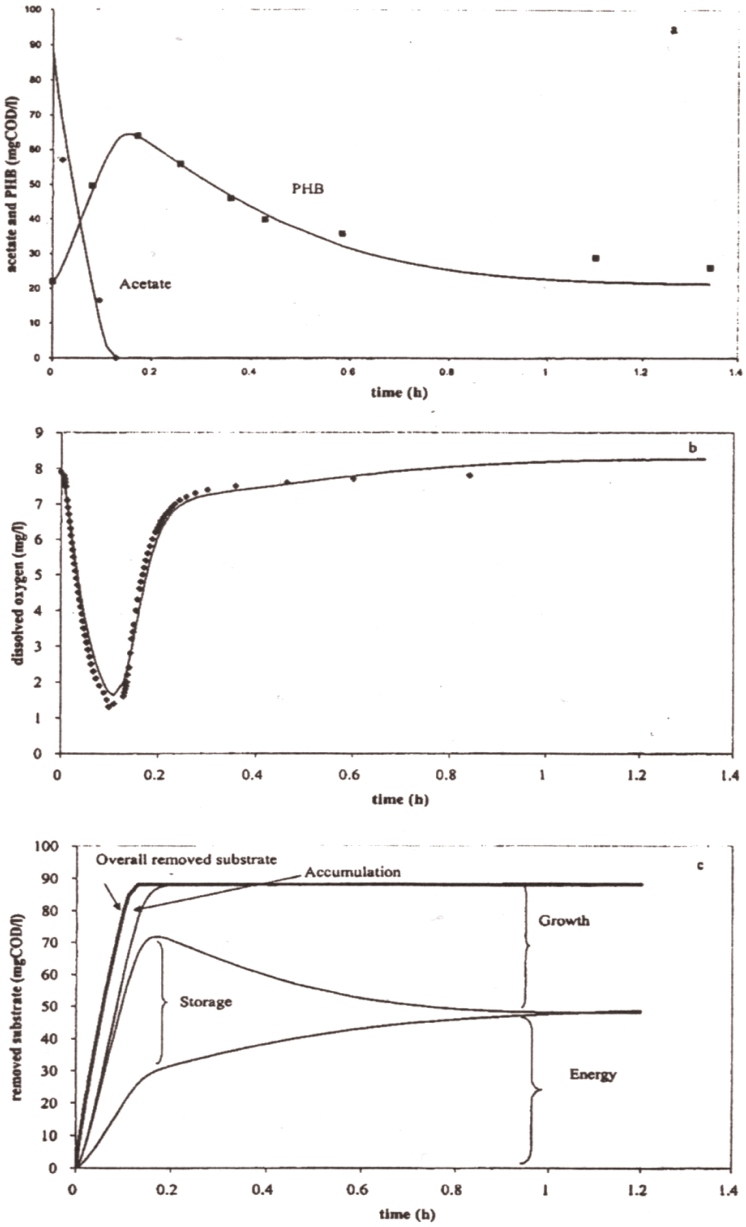


Figure 2.5. Typical SBR cycle under aerobic conditions: (a) acetate and PHB; (b) dissolved oxygen, (c) contribution to the overall COD removed. Solid line refer to simulated profiles according to the kinetic model (Dionisi et al. 2001).

There are more than 300 species known to synthetize PHA and this storage ability is a phylogenetically widespread property (Lee 1996a). However, the knowledge about the genera and species that are enriched under feast and famine conditions is rather limited. In terms of microbial morphology, feast and famine conditions are known to often enrich for floc forming rather than filamentous bacteria (Martins et al. 2004).

Staining techniques such as Nile blue/Nile red can be used to visualize PHA inclusions in microscopy (Serafim et al. 2002).

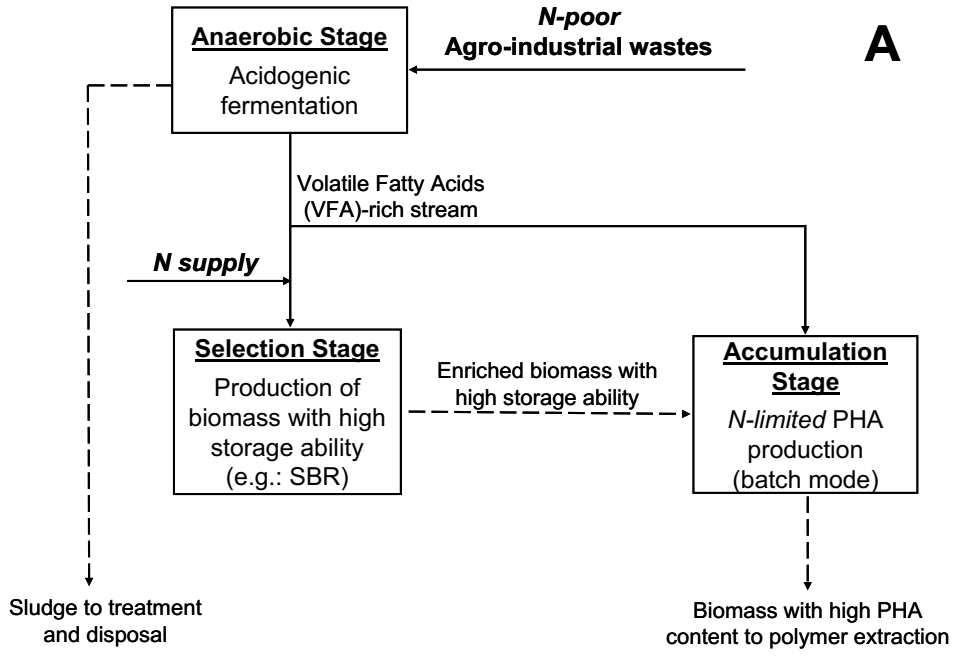
Cultures subjected to feast and famine conditions with acetate as carbon source under ammonia limitation have shown high abundance of the genera *Thauera* and *Azoarcus* (41-49% and 23-46% of bacteria, respectively) with 1 and 10 days SRT (Lemos et al. 2008; Serafim et al. 2006). *Amaricoccus* sp. were highly abundant (61%) with propionate as substrate, abundant with acetate at 10 days SRT (29%) but absent (<1%) with 1 day SRT. These results were obtained by fluorescence *in situ* hybridization (FISH) which, after combination with Nile blue staining, enabled to confirm these organisms as PHA producers.

Abundance of *Thauera* sp. was also shown with denaturing gradient gel electrophoresis (DGGE) for a culture enriched at 1 day SRT on a mixture of acetate, propionate and lactate as carbon source (Dionisi et al. 2005b). Besides, this culture was dominated by *Methylobacteriaceae* sp., *Flavobacterium* sp. and *Meganema perideroedes*. A culture selected with the same carbon source but at higher organic load rate (OLR, up to 31.25 gCOD/l/day) was, according to a 16S rDNA clone library, dominated by *Thauera* sp., *Alcaligenes* sp. and *Comomonas* sp (Dionisi et al. 2006). Another culture selected with a mixture of acetic and propionic acid as carbon source, at an OLR range of 8.5 - 40.8 gCOD/l/day, was dominated by *Lampropedia hyalina* and *Thauera* sp. at the lowest and at the highest OLR respectively (Beccari et al. 2010).

Overall, even though PHA storage from MMCs has been studied in the past mostly in relation to its relevance to wastewater treatment, current research is focusing on the use of various and ever increasing range of waste streams for their commercial production with mixed cultures.

However, some feedstock (such as some agro-industrial wastes) cannot be directly converted to PHA and a preliminary treatment is usually required to convert their organic content into more direct substrates for PHA storage, such as volatile fatty acids (VFAs). As a consequence, the proposed process for mixed culture PHA production usually comprises of three stages (figure 2.6): a first acidogenic fermentation stage, a successive culture selection stage (under feast and famine regime), and a final PHA accumulation stage after which the polymer is extracted

from the cells and purified. Under feast and famine condition, a culture is exposed to alternating substrate availability and unavailability. This conditions can be obtained in an intermittently fed reactor (such as a sequencing batch reactor, SBR), with two reactors in series with biomass recirculation to the first reactor (the selector) or with a plug flow reactor. The performance and efficiency of the overall process largely depends on the nature of the waste used as feedstock and, in particular, its C:N ratio plays a pivotal role. As an example, the use of N-poor waste streams enhances PHA production in the accumulation stage even though it makes the supply of nitrogen sources necessary to sustain biomass growth in the selection stage (figure 2.6A). Conversely, when N-rich wastes are used no external nitrogen supply is needed to select microorganisms with high storage ability but a lower PHA content is usually obtained in the accumulation stage. Otherwise, nutrient limiting conditions can be established in the accumulation stage by an additional step for nutrient removal from the medium (figure 2.6B).



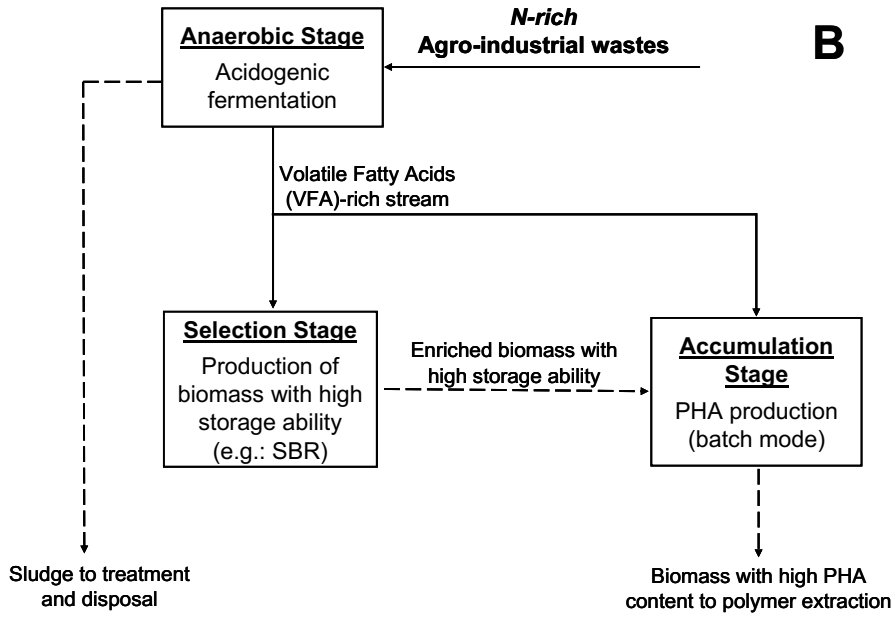


Figure 2.6. Outline of a three-stage process used for PHA production from N-poor (A) or N-rich (B) agro-industrial wastes by employing mixed microbial cultures selected under feast and famine regime (solid lines refer to liquid fluxes and dotted lines to biomass fluxes).

2.3.2 Feast and famine condition: metabolism

The methabolic pathways for polymer synthesis in mixed cultures submitted to feast and famine conditions have been assumed to be similar to those of pure cultures (figure 2.7).

Acetyl-CoA is the central metabolite that is formed from acetate. Acetyl-CoA is partially channeled to the tricarboxylic acid cycle for production of biomass precursors, energy equivalents (ATP) and reducing equivalents (NADPH) and partially to form PHA monomers. Two acetyl-CoA units can be combined and reduced to a 3HB monomer. Propionate is activated to propionyl-CoA which can be combined with one unit of acetyl-CoA to form 3-hydroxyvalerate (via 3-hydroxyvaleryl-CoA) or to 3-hydroxy-2-methylbutyrate (via 3-hydroxy-2-methylbutyryl-CoA). The latter monomer is usually formed to a much lesser extent. Propionyl-CoA can also undergo decarboxylation to acetyl-CoA. Two units of propionyl-CoA can be combined to form one monomer of 3-hydroxy-2-valerate. Production of this monomer has only been observed with mixed cultures and, so far, not with any pure culture (Michinaka et al. 2007).

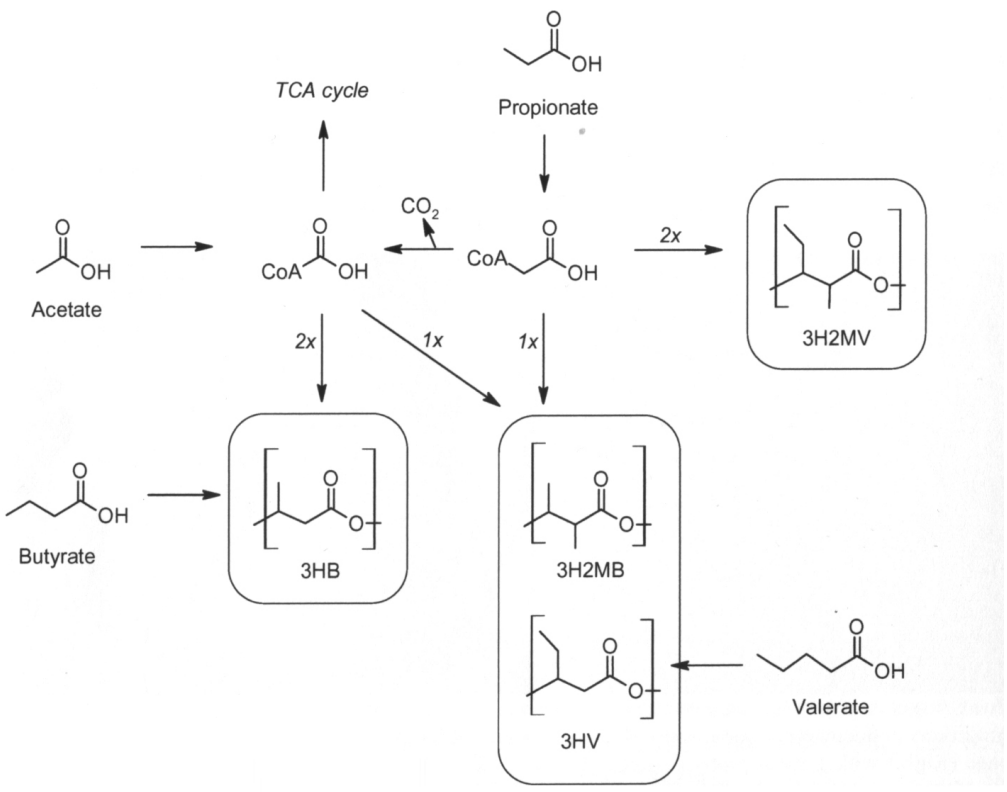


Figure 2.7. Representation of PHA production from different VFAs. Some of the reaction involve more than one metabolic step.

Butyrate and valerate can be directly converted to 3-hydroxybutyryl-CoA and 3-hydroxyvaleryl-CoA for formation of 3HB and 3HV, respectively.

A metabolic model has been developed for the case of acetate as sole carbon source with a pure culture of *Paracoccus pantrophus* (van Aalst-van Leeuwen et al. 1997) and adapted for mixed cultures (Beun et al. 2002; Beun et al. 2000; Dias et al. 2005). Seven reactions are considered for feast (storage, growth and maintenance on acetate) and famine conditions (growth and maintenance on PHB). Based on these metabolic reactions, material balances of biomass precursors, $NADH_2$, ATP and acetyl-CoA and overall degree of reduction balances can be used to derive equations for the calculation of yield and maintenance coefficients as a function of the efficiency of oxidative phosphorylation (δ or P/O in mol ATP per mol $NADH_2$). In this way substrate uptake for growth, PHB formation and maintenance could be described by a linear equation. The maximum yield of PHB on acetate (in absence of

biomass growth) was determined to 0.65 C-mol/C-mol (δ = 1.84 mol ATP/mol $NADH_2$) for *Paracoccus pantrophus* (van Aalst-van Leeuwen et al. 1997) and 0.74 C-mol/C-mol (δ = 3.0 mol ATP/mol $NADH_2$) for a mixed culture enriched under feast and famine conditions (Dias et al. 2005). This modeling approach was recently extended to the case of acetate and propionate as substrate and used to described production of polymers containing 3HB, 3HV and 3H2MV (3-hydroxy-2-methylvalerate) by two cultures enriched with acetate and propionate as sole carbon sources (Dias et al. 2008).

With acetate as sole carbon source, a homopolymer of P(3HB) is obtained. However, the composition of the produced PHA depends both on characteristic of the enriched culture and on the substrate used. The metabolic characteristic of the enriched culture depend mainly on the carbon substrate used for enrichment. This was shown by feeding acetate and propionate, as sole carbon source and in combination, to two cultures enriched with acetate and propionate, as sole carbon source (Lemos et al. 2006). It was shown that feeding acetate to the culture enriched with propionate resulted in the production of 3HV (17 mol-%) and 3HB. Feeding propionate to the culture enriched with propionate resulted resulted in 3HB and 3HV (3HB:3HV molar ratio 15:85) and feeding propionate to the culture enriched with acetate resulted in 3HB, 3HV and 3H2MV (3HB:3HV:3H2MV molar ratio 6:58:24). Also when feeding a mixture of acetate and propionate, the two cultures responded differently. Whereas the culture acclimated to acetate produced 3HB, 3HV and 3H2MV, the culture acclimated to propionate produced 3HB and 3HV, with a higher fraction of 3HB instead.

A mixed culture adapted to a mixture of acetate and propionate was found to produce 3HB and 3HV with an increasing fraction of 3HV up to 84% mol-% 3HV with pure propionate (Takabatake et al. 2000). Based on that *Cuparidus necator* produces around 40 mol-% 3HV from pure propionate (Doi et al. 1987), it was concluded that a mixed cultures can produced PHA with a wider range of 3HB:HV from acetate and propionate. A mixed culture adapted to a mixture of acetate, propionate and lactate produced pure 3HV when propionate was the sole carbon source (Dionisi et al. 2004). It was also found that lactate was converted to 3HB by this mixed culture which is also the case for *C. necator* (Tsuge et al. 1999).

The polymer yield on substrate is typically lower for propionate than for acetate, especially when propionate is the sole carbon source (Dionisi et al. 2004; Lemos et al. 2006). This is since in the formation of 3HB and 3HV from propionate, propionyl-CoA will have to be converted to acetyl-CoA by decarboxylation. Interestingly, mixed cultures do not appear to selectively adapt to propionate by accumulating higher amount of 3H2MV.

Thus, it may appear that the polymer composition is not easy predictable from a given VFA feed since it also depend on the enrichment substrate. With the additional assumption that lactate is converted to 3HB, this approach was also successful in predicting the composition of 3HB:3HV produced from a synthetic mixture of acetate, propionate and lactate (Dionisi et al. 2004).

The kinetics of PHA accumulation and degradation are related to the intracellular PHA level. As long as culture has a low intracellular PHA content, the rate of PHA accumulation is high. If the PHA content reaches higher levels, the rate will decrease with increased PHA content. Therefore, the rate of PHA production is mathematically described with a saturation expression with respect to the PHA cell content (Dias et al. 2005; van Aalst-van Leeuwen et al. 1997). The rate of PHA degradation under feast and famine conditions has also been observed to be dependent on PHA cell content. The kinetics of PHA degradation is described by either first (Beun et al. 2002; Carta et al. 2001; Serafim et al. 2004) or n^{th} order (Beun et al. 2000; Dias et al. 2005; Dircks et al. 2001) functions of the PHA cell content.

2.4 Recovery techniques for the isolation and purification of PHA from microbial cells

The high production cost of PHA has limited their market diffusion. The major cost absorbing factors are the upstream fermentation processes and the downstream PHA recovery technologies. The latter significantly affects the overall process economics. Various recovery technologies have been proposed and studied in small lab-scales as well as in industrial scales. These include solvent extraction, chemical digestion, enzymatic treatment and mechanical disruption, supercritical fluid disruption (emerged PHA extraction technique), flotation techniques.

2.4.1 Solvent extraction

Solvent extraction is the most extensively adopted method to recover PHA from the cell biomass, also used routinely in the laboratory because of its simplicity and rapidity (Kunasundari and Sudesh 2011). In addition, is an high efficiency method with a negligible degradation to the polymers (Jacquel et al. 2008), thus, it is possible to obtain very pure PHA with high molecular weights. The first step involved is the modification of cell membrane permeability thus allowing release and solubilization of PHA, followed by non-solvent precipitation (Jacquel et al. 2008). Chlorinated hydrocarbons (i.e. chloroform, 1,2-dichloroethane; Ramsay et al. 1994) are the most utilized. Precipitation of PHA is commonly induced by non-solvent such as methanol and ethanol (Ramsay et al. 1994).

Unfortunately, large-scale application of solvent extraction is not and environmentally friendly methods. Thus, 1,2-propylene carbonate has been proposed as an alternative to halogenated solvents in the recovery process of PHA (McChalicher et al. 2010; Fiorese et al. 2009). Higher boiling point (240°C) of 1,2-propylene carbonate prevents the evaporation to the environment at lower temperatures and allows its reusability for several cycles of purification. This could reduce the solvent consumption and therefore it is viewed as

economically advantageous. Besides, 1,2-propylene carbonate is considered safe due to its low toxicity. Fiorese et al. (2009) reported a maximum PHA yield of 95% and a purity of 84% when extracted from the *C. necator* cells at 130°C for 30 min without involving any pretreatment. This is comparable to the values obtained from chloroform extraction (94% yield and 98% purity).

2.4.2 Chemical digestion

The approach is based on the solubilization of non-PHA cellular mass (NPCM) and mainly utilizes sodium hypochlorite or surfactants. The important features of sodium hypochlorite such as strong oxidizing properties and non-selectivity can be manipulated to digest NPCM and facilitate PHA recovery (Yu and Chen, 2006). As surfactants, sodium dodecyl sulfate (SDS) was the most evaluated.

Isolation of PHA granules by surfactant digestion exhibited lower degree of purity with higher molecular weight than sodium hypochlorite digestion (Kunasundari and

Sudesh 2011). In contrast, PHA of higher purity was obtained using sodium hypochlorite but with severe degradation of molecular weight up to 50% (Ramsay et al. 1990). Sequential surfactant-hypochlorite treatment promoted better and rapid recovery of PHA (Ramsay et al. 1990; Dong and Sun, 2000) and resulted in 50% reduction of overall cost when compared to solvent extraction (Yu, 2009). Despite of the low operating cost (Jacquel et al. 2008) and technical simplicity, the problems relative to the surfactant in wastewater treatment and relatively high cost of chemical agents such as SDS and sodium hypochlorite, are still unresolved.

Another method is the selective dissolution of non-PHA cell mass by protons (Yu and Chen, 2006; Yu, 2009). The most important step is the solubilization of NPCM in acid solution to release partially crystallized PHA granules and later subjecting the suspension to decolorization in a bleaching solution. This method was claimed to lower the recovery cost by using cheaper chemicals with higher recovery efficiency. However, the process parameters have to be controlled in order to maintain the molecular weigth at a minimum of 50% of the original.

2.4.3 Enzymatic digestion

Recovery process of PHA using enzymatic digestion involves a rather complex procedure, however this technique is attractive because of its mild operation condition (Middelberg, 1995; Kapritchkoff et al. 2006). Solubilization of cell components other than PHA typically consists of heat treatment, enzymatic hydrolysis and surfactant washing (Holmes and Lim, 1990). Lakshman and Shamala (2006) used *Microbispora* sp. culture, which was identified to secrete protease, in the fermented broth of *S. meliloti* containing 50% of PHA to induce hydrolysis. The culture was introduced to the thermally (80°C for 10 min) inactivated biomass of *S. meliloti* and incubated for 72 h. The *S. meliloti* cells were hydrolyzed by the protease resulting in the release of the intracellular components together with the PHA granules. The culture containing the lysed cells was then subjected to a simple filtration process and PHA of 94% purity was recovered using chloroform extraction. Because enzymes are very specific with respect to the reactions they catalyze, recovery of PHA with good quality could be expected. Nevertheless, the high cost of

enzymes and complexity of the recovery process outweigh its advantages (Kapritchkoff et al. 2006).

2.4.4 Mechanical disruption

Among the various mechanical disruption methods, bead milling and high-pressure homogenization dominate the large scale cell disruption in pharmaceutical and biotechnology industries (Bury et al. 2001). Unlike other recovery methods, mechanical disruption is favored mainly due to economic advantageous and because it causes mild damage to the products (Tamer et al. 1998). Mechanical disruption of cells does not involve any chemicals so it minimizes environmental pollution (Jacquel et al. 2008) and contamination to the products (Tamer et al. 1998). In general, the drawbacks of mechanical disruption method are, high capital investment cost, long processing time and difficulty in scaling up (Park et al. 2007; Balasundaram and Harrison, 2008). Mechanical cell disruption is widely used to liberate intracellular protein (Harrison, 1991). The concept has been tested to recover PHA from bacterial cells (Tamer et al. 1998).

2.4.5 Supercritical fluids (SCF)

Supercritical fluids (SCF) have emerged as a potential PHA extraction technique (Hejazi et al. 2003; Khosravi-Darani and Mozafari, 2009; Khosravi-Darani et al. 2003) based on the unique physicochemical properties of SCF (high density, low viscosities). Supercritical-carbon dioxide (SC–CO_2) is the most predominantly used SCF, characterized by low toxicity and reactivity, moderate critical temperature and pressure (31°C, 73 atm), availability, low cost, non-flammability (Hejazi et al. 2003). By using this method, P(3HB) recovery of 89% from *C. necator* at 200 atm, 40°C and 0.2 ml of methanol was reported (Hejazi et al. 2003). However, SC–CO_2 efficiency in bacterial cell disruption is very much dependent on the process parameters such as operating pressure, temperature, culture cultivation time (Khosravi-Darani et al. 2004). High temperature and pressure significantly influence the physiological properties of cell membrane that prevent the biopolymer from being extracted. Khosravi-Darani et al. (2004) investigated on a series of pretreatment to improve the SC–CO_2 disruption: with 1% (v/v) toluene as a modifier,

200 bar of pressure, 30°C temperature and two times SC–CO_2 pressure release, up to 81% P(3HB) recovery could be achieved by using wet cells of *C. necator*. The proposed PHA recovery process could be economically feasible taking into account that the lyophilisation step could be avoided.

2.4.6 Flotation

Ibrahim and Steinbüchel (Ibrahim and Steinbüchel, 2009) investigated the recovery of P(3HB) from a recently isolated bacterium, *Zobellella denitrificans* MW1. Simple extraction with various organic solvents followed by self-flotation of cell debris was tested. The cells were mixed with chloroform at 30°C for 72 h and later subjected to self-flotation of cell debris overnight at room temperature. This method allowed efficient recovery of 85% (w/w) of P(3HB) with purity of 98%. Adoption of green solvents together with flotation technique perhaps would add more benefits for the downstream processing. Previously, selective dissolved-air flotation was also applied to extract mcl-PHA from the cell debris of *P. putida* (van Hee et al. 2006). The main limitation of dissolved-air flotation is that it requires several consecutive flotation steps.

2.4.7 Aqueous two phase system (ATPS)

Another potential method for the recovery of PHA was recently reported using *B. flexus* (Divyashree et al. 2009). Aqueous two phase system (ATPS) is formed when two polymers at low concentrations (or of one polymer and an inorganic salt) that display incompatibility are mixed such that two immiscible phases coexist (Yang et al. 2008). PHA containing *B. flexus* cells were subjected to enzymatic hydrolysis of *Microbispora* sp. cells filtrate and later introduced to polymer-salt ATPS system (Polyethylene glycol [PEG] 8000/phosphate, pH 8.0 and 28°C) reported recovery of high molecular weight PHA ($1 \cdot 10^6$) with 97% purity. Several factors, i.e. polymer molecular weight, concentration of polymer and salt, pH, molecular mass, charge etc. have to be considered to choose a good ATPS recovery system. This technique is considered attractive because of short processing time, low material cost, low energy consumption, good resolution, high yield and a relatively high capacity (Kunasundari and Sudesh 2011; Yang et al. 2008; Rito-Palomares, 2004).

In conclusion, in order to obtain PHA with a high degree of purity, more stringent recovery process is needed that often results in PHA with lower molecular weights and recovery yield. Therefore, the challenge in the recovery process is to maintain the original molecular weights (regardless of PHA final applications, the molecular weights of the recovered product should be sufficiently high), while not compromising the degree of purity for various applications (Kunasundari and Sudesh 2011). These criteria have to be achieved in an environmentally friendly manner. Finally, the cost of the recovery process should be economically feasible. Together with PHA recovery techniques, fermentation strategies also should be governed. Development of strains that could effectively overproduce PHA from various unprocessed, cheap and renewable carbon sources also plays an important role in lowering the cost of PHA production (Kunasundari and Sudesh 2011; Akaraonye et al. 2010).

CHAPTER 3. AIMS

3.1 Background

In the second stage of the process, operating conditions affect the community composition of the enriched culture. This stage should be optimized to produce a biomass with a high and stable potential for PHA storage. A stable potential both with respect to fraction of PHA in the biomass and the PHA properties is desiderable. In the third stage of the process, operating conditions have a direct effect on the PHA produced. This stage should be optimized for a targeted and stable polymer composition and for maximum PHA biomass content, yield and production rate. Outcomes of PHA accumulation depend on the microbial composition of the enriched culture and therefore optimization of these two stages are not independent.

Most of the studies on PHA production by mixed cultures are based on the use of VFAs as the carbon source (Beccari et al. 1998; Serafim et al. 2004; Dionisi et al. 2004; Lemos et al. 2008) because they are predominant products in pre-fermented carbohydrates (Albuquerque et al. 2007; Bengtsson et al. 2008). In the second stage, an activated sludge process is operated at high organic load rate (OLR) by periodic feeding in a SBR, in order to enrich and produce a sludge with high storage response. In establishing the selection in favor of a biomass with high storage rates through the alternation of feast and famine conditions, the OLR plays an important role. Most reported studies (Beccari et al. 1998; Beun et al. 2000; Beun et al. 2002; Martin et al. 2003; Serafim et al. 2004) focused on storage response of mixed cultures were carried out in the lower OLR range (approximately 0.3 - 1.2 gCOD L^{-1} d^{-1}), showed that the feast and famine conditions were always established and the developed biomass exhibited a good storage response. However, in order to increase the volumetric productivity in mixed culture PHA production, higher OLR are being aimed.

The effect of significantly higher OLR on the biomass selection was investigated too (Dionisi et al. 2004; Dionisi et al. 2005b; Dionisi et al. 2006; Beccari et al. 2010). Dionisi et al. (2004) operated an SBR fed with a mixture of acetate, lactate and propionate (40:40:20 % on a COD basis) at 1 day SRT and 8.5 gCOD L^{-1} d^{-1} as OLR. The obtained PHA content was 45% of TSS which is lower than has been obtained

applying lower OLR and higher SRT with acetate, 65% (Serafim et al. 2004). The effect of OLR in the range 8.5 – 31.25 gCOD L^{-1} d^{-1} (by increasing the feed concentration) was studied with a fixed SRT of 1 day and it was shown that a storage response could be obtained up to 20 gCOD L^{-1} d^{-1} (Dionisi et al. 2005b). Above this OLR, the feast phase became longer, which lead to a decrease in the famine phase duration and therefore the selection pressure was lost and microorganisms with growth response was selected (figure 3.1). In fact, a strong selective pressure for PHA storage is related to the feast/famine regime (Majone et al. 1996; Dionisi et al. 2006; Dionisi et al. 2007; Johnson et al. 2009; Beccari et al. 2010; Albuquerque et al. 2010) to which the cultures are exposed. The PHA productivity in a three stage process (which depend on both biomass concentration and polymer production rate), reached a maximum value of about 6 gPHA L^{-1} d^{-1} at OLR of 20 gCOD L^{-1} d^{-1}, even if an unstable storage response was obtained at this OLR in SBR as reported in figure 3.1.

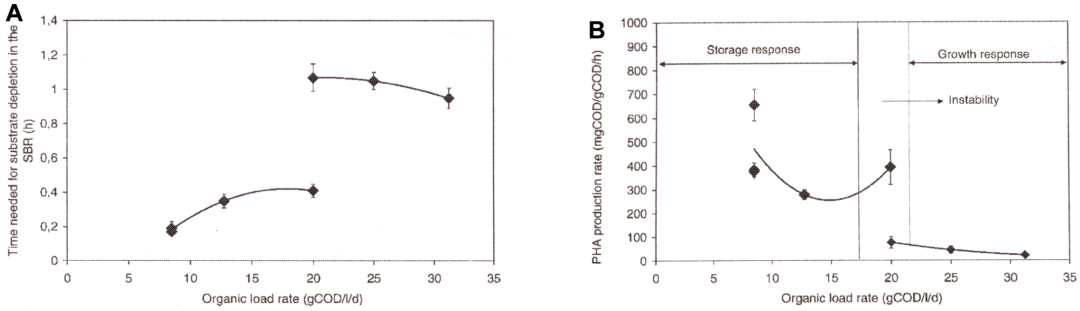


Figure 3.1. (A) Time needed for substrate depletion (i.e. feast phase); (B) Polymer production rate in accumulation tests with biomass acclimated at different OLR. (Dionisi et al. 2005b).

Further (Beccari et al. 2010), the SBR was operated up to OLR of 40.8 gCOD L^{-1} d^{-1}. The increasing of OLR was obtained, differently from previous study, by decreasing the operating cycle length (from 2 to 0.42 h). The feed concentration was the same for all the experiments (8.5 gCOD L^{-1} acetate and propionate 85:15 % as COD); as a results, also the HRT changed from 1 to 0.21 day. Both parameter (OLR and HRT) affected the establishment of the feast/famine condition. The best storage response was obtained at the lowest OLR investigated and highest HRT (8.5 gCOD L^{-1} d^{-1}; HRT 1 day) (figure 3.2). However, a clear and stable storage response was maintained at least up to 25.5 as OLR, where the polymer productivity was the

highest recorded (~2.81 gPHA L^{-1} d^{-1}). Despite of the best storage performance, in terms of final PHA content, obtained at the lowest OLR investigated, by increasing the OLR or decreasing HRT (or SRT), the biomass productivity increased, involving in a higher PHA productivity and in a lower PHA content. The best choose was a compromise between these two important aspects.

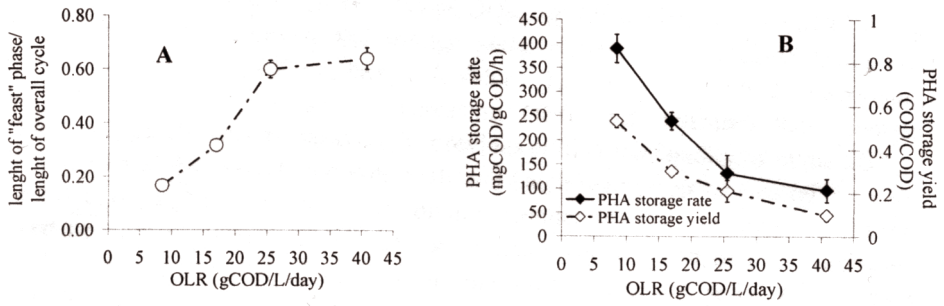


Figure 3.2. Effect of the applied OLR on the ratio between the length of the feast phase of the cycle (A), and on PHA storage rates and yields in SBR (B). (Beccari et al. 2010).

Moreover, no nutrients limitation was imposed in these process implemented (Dionisi et al. 2004; Dionisi et al. 2005b; Beccari et al. 2010), especially in the third stage, where a growth limitation could lead to an increase of storage phenomena and to a maximal PHA saturation capacity of the biomass.

Enrichment of a culture with a high storage potential is feasible both under nutrient excess (Beccari et al. 1998; Takabatake et al. 2000; Bengtsoon et al. 2008) and under nutrient limitation (Lemos et al. 2006; Serafim et al. 2004). In this context, the nature of the organic substrate also plays a role: selection of a PHA storing culture was not successful applying the same organic loading rate and nutrient levels as Serafim et al. (2004) but with fermented molasses instead of synthetic acetate as substrate (Albuquerque et al. 2007). In this case, excess nutrients were necessary for selecting a PHA storing culture. Excess nutrients result in a higher biomass yield which positively affects the overall polymer productivity over the three-stage process. For culture selected at very high growth rates, such as 1 day SRT, the influence of nutrient limitation on PHA storage yields and rates is less pronounced (Dionisi et al. 2005b).

A study of Dionisi et al. (2007) also showed the relevant role of the length of the cycle on storage response of the selected biomass at fixed and relatively high OLR (20 gCOD L^{-1} d^{-1}). The selected biomass was enriched with microorganisms that were able to store PHA at high rates and yields only when the length of the cycle was 2 or 4 h, even though in these conditions the process was unstable. At a cycle length of 1 or 8 h, the dynamic response of the selected microorganisms was dominated by growth. The corresponding productivity of the process was in the range 6-7.2 gPHA L^{-1} d^{-1}, quite high for a mixed cultures system. Despite of the importance of this parameter, limited research has been devoted to the effect of operating cycle length.
These results enriched the background of knowledge for a further investigation on the choice of the value of the principal operating parameter in a perspective of process optimization. Thus, in order to achieve a good and stable storage performance, the SBR has to be operated at OLR higher than in traditional activated sludge systems. On the other hand, if the OLR is too high, the strength of the feast and famine regime can be diminished. Moreover, the increase in OLR can be in principle an advantage for the process PHA productivity, as it increases the biomass productivity. However, it has to be verified whether the produced biomass maintains high storage rates also at higher loads, suggesting the existence of an optimal OLR to be found.

3.2 Structure and purposes of the experimental plan

The research was divided in 3 fundamental steps:

- Based on the lack of knowledge related to the biomass behavior in the acclimation phase, under new dynamic feeding condition in SBR (feast/famine), the process performance in the early days after SBR start up was firstly investigated, by taking the biomass from SBR and performing the third accumulation stage in batch, under pulse feeding condition. 4 SBR runs characterized by short duration (8-10 days maximum) were implemented at the same operating condition starting from fresh inocula sampled from wastewater treatment plant: OLR gCOD L^{-1} d^{-1}, HRT 1 d, overall cycle length 2 h, pH 7.5 and T 25°C. A synthetic mixture of acetic and propionic acid (85:15 % on a COD basis) was used for both SBR and batch tests. A

nitrogen limitation in batch tests was imposed in order to prevent the cellular growth maximizing the storage capacity of the biomass. The storage performance obtained with this partial acclimated activated sludge was compared to that obtained with fully acclimated biomass (characterized by longer acclimation period to the feast/famine regime, 160 days), in terms of specific storage rate and yield, PHA content and productivity.

- Then, the documented and more traditional approach (Dionisi et al. 2001; Dionisi et al. 2004; Dionisi et al. 2005b; Dionisi et al. 2007; Beccari et al. 2010) has been used following the biomass behavior in SBR for longer period (90 days at least). Two weeks resulted quite enough in order to obtain a stable performance; after that a good and lasting storage response in SBR was reached (short feast phase) and the accumulation stage was carried-out. In this context, three long-term SBR runs were performed at fixed OLR (8.5 gCOD L^{-1} d^{-1}) and HRT (1 d) pH (7.5) and T (25°C) by using a synthetic mixture of acetic and propionic acid (85:15 % on a COD basis); the effect of the frequency of the alternation of the feast and famine phases was investigated by changing the overall cycle length (2, 6 and 8 h). In the latter case the feeding time was changed in order to evaluate any eventual effect of the maximal substrate concentration at which the biomass was exposed at fixed feeding frequency. In a perspective of a complete process implementation (at least for both aerobic stages of selection/enrichment and accumulation), the effect of an additional starvation time imposed to the biomass, acclimated in SBR operating with a cycle length of 2 h, was investigated in a short-term batch tests.

  Microbial community analysis, based on denaturing gradient gel electrophoresis (DGGE) was carried out in order to evaluate if the cycle length plays a main role on the microbial speciation within the SBR.

- Based on the results obtained from the three long-term SBR run, a continuous system consisting in II and III stage (the aerobic steps of the PHA production process) was developed at OLR 8.5 gCOD L^{-1} d^{-1}, HRT 1 d, pH 7.5, T 25°C and overall cycle length 6 h in SBR. The SBR was fed with a synthetic

mixture of acetic and propionic acid (85:15 % COD basis). The biomass withdrawn from SBR, at each cycle, was collected in the batch aerobic reactor fed with a more concentrated synthetic solution (~44 gCOD L^{-1} 85:15 % COD basis) respect to the SBR feeding: this was necessary in order to significantly increase the S/X ratio in batch than SBR, taking into account that the biomass was not diluted when discharged from SBR to batch reactor. After an accumulation stage of 6 h, the same time of SBR cycle, presumably sufficient to enrich the polymer saturation (the accumulation stage was carried out under nitrogen limitation condition), the biomass was continuously collected and treated with quenching reagent for PHA extraction; then the stored polymer was purified and characterized via lyophilisation and $CHCl_3$ treatment.

The last part of the experimental program consists in the optimization of the accumulation stage in terms of nutrient content of wastewaters used as substrate. In particular, the nitrogen and phosphorous concentration effect was exploited in batch tests conducted with acclimated biomass sampled from a pilot-scale SBR in Lund (AnoxKaldnes). In the synthetic feed (consisting of acetic acid as only carbon source), the COD/N and COD/P ratios covered a wide range of values, changing from starvation to excess condition in the growth medium.

CHAPTER 4. BIOMASS ACCLIMATION, ENRICHMENT AND PRODUCTION IN THE II STAGE THROUGH SBR

4A. Start up of a biological SBR and short-term biomass acclimation for PHA production

With reference to PHA-accumulating culture selection in SBR, previous studies were focusing on the mechanisms for increased selective pressure for PHA storage; the existing literature supports the hypothesis of a strong pressure for PHA storage being related to the feast/famine to which the cultures are exposed.

Johnson et al.(2009) maximized the selective pressure imposed on the culture selection SBR (using an influent acetic acid concentration of 54 Cmmol L^{-1}) by gradually decreasing the sludge retention time (SRT) to 1 d and gradually increasing the operating T to 30°C. The authors obtained a culture with a high storage capacity (89% PHA content under nitrogen limitation during the accumulation step). The relative low SRT and high T are suggested by the authors to be, at least, partially responsible for the high kinetic parameters reported. In addition, the influent substrate concentration resulted in a very low feast phase (9% of the overall cycle length).

Dionisi et al.(2006) tested the effect of the OLR (through change in influent substrate concentration) on the performance of an SBR fed with a mixture of synthetic organic acids (lactic, acetic and propionic acids) at very high OLR in the range of 8.5-31.25 gCOD L^{-1} d^{-1}. The feast phase length reported by the authors were in the range 11.5-59% of the cycle length; an high storage response was obtained for less high OLRs (feast phase up to 22.5%), a growth response for higher OLRs (feast phase higher than 50%) and a transient response at an intermediate value in which a very unstable system was selected (feast phase varying from 22.5 to 50%).

In a later study (Beccari et al. 2010) the authors operated the SBR (fed with a synthetic mixture of acetic and propionic acid) at very high OLR, in the range 8.5-40.8 gCOD L^{-1} d^{-1}, and very short HRT in the range 1-0.21 day, both being simultaneously varied by changing the cycle length from 2 h to 0.42 h. Both parameters affected the establishment of the feast and famine conditions. It was observed that, at an increasing of applied OLR, the feast phase increased from 16.7%

to 64%, with a progressive loss of the selective pressure required to select microorganisms with high storage response. Indeed, a decrease of both PHA storage rate and yield was observed; the highest values being obtained at the less high OLR investigated (389 mgCODgCODnonPolym^{-1}h^{-1} and 0.53 COD COD^{-1}, respectively).
In a study of Albuquerque et al. (2010) the tested OLRs were in the lower values range (2.2-4.4 gCOD L^{-1} d^{-1} using fermented real wastewater). The range of feast phase length observed was 17% - 50%; consistent with the findings of Dionisi et al. (2006); the lowest OLR resulted into feast length of 17% and was associated with a predominantly storage response, while a very unstable system (highly dynamic) with alternate predominantly storage and growth responses was obtained for a higher influent substrate concentration; the resulting feast phase length varying for the same average loading from 34.6% to 52% of the overall cycle length.
The SBR is usually inoculated by an activated sludge from wastewater treatment plants, whose ability to produce PHA is progressively increased through several mechanisms (physiological adaptation, selection and enrichment). Thus, the quickness of activated sludge adaptation before reaching high PHA storage response is also a key factor for process design and operation as well as for process control, also taking into account possible disturbances from the steady state. Very few studies have been focus on the acclimation phase of the biomass under new dynamic feeding conditions and new types of substrate, both different from wastewater treatment plants. Mengmeng et al. (2009) investigated the storage capacity of no-acclimated biomass taken from the municipal wastewater treatment system. Their experimental plan did not include any acclimation or enrichment stage, as usually done in order to obtain a good selection of biomass with high storage response. The biomass consisted in excess sludge sampled from the secondary sedimentation tank of a wastewater treatment plant; it was directly inoculated into an aerobic batch reactor where an excess sludge fermentative liquid (characterized by high VFAs content) was used as substrate. The sludge accumulated PHA immediately and the intracellular PHA content increased from 3.5% to 26.7% of the dry cell before the second substrate feeding. This indicated a quick biomass adaptation to the new conditions. Spitting the substrate in three pulses led to 56.5% of PHA content at the time of total substrate depletion (3 h and 40 minutes). This saturation value,

confirmed by no increasing of PHA content observed after the fourth pulse, was obtained by using real wastes as carbon source and activated sludge as inoculum without sludge acclimation.

Based on the lack of knowledge related to the biomass behavior in the acclimation phase, under new dynamic feeding condition in SBR (feast and famine), in the present study we investigated the process performance in the early days after SBR start up (from two to ten days from the SBR inoculation) by taking the biomass from the SBR during start up and performing the third accumulation stage in batch experiments under pulse feeding condition. A molecular analysis of the microbial composition of the mixed culture which developed in a long-term SBR run was also carried out by means of denaturing gradient gel electrophoresis (DGGE) and PCR amplification of the bacterial 16S rRNA genes. This was done in order to evaluate any changes occurring in the structure of the bacterial community throughout the run, from the start-up to the end.

4A.1 Materials and methods

4A.1.1 Acclimation and selection stage (SBR Run)

The aerobic enrichment of the PHA-producing biomass was performed in a 1 L working volume SBR that was inoculated with the activated sludge from the "Roma Nord" full-scale wastewater treatment plant (WWTP). Before each inoculum the sludge was left to settle in order to remove the supernatant, and resuspended in a synthetic mineral medium (whose the composition is given below). Then, the sludge was aerated for at least one night before the inoculum; an average volatile suspended solid (VSS) concentration of 4 g L^{-1} was measured.

After each inocula, the SBR was fed with a mixture of acetic and propionic acid (85% and 15% on a COD basis, respectively), at an overall concentration of 8.5 gCOD L^{-1}. The SBR was operated at 12 cycles per day (cycle length 2 h); the substrate feeding and the mixed liquor discharge were made for the first 10 minutes and for last 1 minute of each cycle. Overall, the SBR was operated at applied organic load rate (OLR) 8.5 gCOD L^{-1} d^{-1} and Hydraulic Residence Time (HRT) of 1 d. No settling phase was performed, and all excess biomass was withdrawn along with the

mixed liquor. In this way, the sludge retention time (SRT) was equal to hydraulic retention time (HRT of 1 d).

Four SBR runs were sequentially carried out at the same operating conditions and for a relatively short period (20 days at the maximum). Furthermore, an additional run was started, at the same operating conditions of the previous but characterized by a much longer period (more than 150 days) in order to reach a complete sludge acclimation to the feast-famine regime, to check process stability and to compare the storage performance with the shortly acclimated sludge. The inocula were sampled from the wastewater treatment plants and used fresh at each time the reactor was started again. The reactor was stirred by a mechanical impeller and aerated by means of membrane compressors (in order to maintain aerobic condition). In working days, the reactor was daily cleaned during the famine phase, by passing manually a spatula on the walls, in order to prevent the biofilm formation and obtain most homogeneous conditions inside the reactor. The reactor cleaning was not performed during the week-end, this involved in some biomass growth on the SBR walls.

The organic feed was prepared in a mineral medium, all the elements being present in excess of biomass requirements for growth (thus preventing any external limitation to growth). The mineral medium concentration was (mg L^{-1}): $(NH_4)_2SO_4$ (3000), $FeCl_3 \cdot 6H_2O$ (2), Na_2EDTA (3), K_2HPO_4 (330), KH_2PO_4 (260), $MgSO_4 \cdot 7H_2O$ (100), $CaCl_2 \cdot 2H_2O$ (50), $ZnSO_4 \cdot 7H_2O$ (0.1), $MnCl_2 \cdot 4H_2O$ (0.03), H_3BO_3 (0.3), $CoCl_2 \cdot 6H_2O$ (0.2), $CuCl_2 \cdot 2H_2O$ (0.01), $NiCl_2 \cdot H_2O$ (0.02), $Na_2MoO_4 \cdot 2H_2O$ (0.03). Thiourea (20 mg L^{-1}) was also added to inhibit nitrification. The pH was maintained at 7.5 by dosing CO_2, which was suitably added via a control system. The temperature was maintained at 25°C by using thermostatic bath.

During each SBR cycle, the dissolved oxygen (DO) was continuously monitored and recorded by using DO probe (CellOx 325 WTW). Aeration and stirring were high enough to maintain DO above 2 mg L^{-1} along the whole cycle. During the feeding, the DO concentration decreased to a low and almost constant value; the following increase of DO concentration indicated that the substrate had been depleted. This point also indicated the end of the feast phase duration and the start of famine phase and was used to calculate the feast-famine ratio for each cycle. The SBR performance was also monitored by measurement of biomass concentration, as

volatile suspended solid (VSS, sample taken at the end of the feast phase), and of PHA (samples taken at the end of both the feast phase and of the famine phase). At the end of the feed the maximum value of substrate concentration within each cycle was obtained, whereas the time of substrate depletion corresponded to the maximum value of polymer concentration. Sampling was usually done once per day.

4A.1.2 Batch accumulation tests

For each short SBR run, two or three short-term aerobic batch test were performed in the period ranging from 2-10 days from the start-up, (i.e., presumably when biomass was not completely acclimated). For the longest SBR run, two similar batch tests were conducted after approximately 150 days, more than adequate period to achieve a stable operating condition process and a high biomass storage response. All the batch tests were performed with multiple and instantaneous spikes of substrate in order to obtain a feast phase quite longer than in the SBR and so to determine the maximal specific PHA storage rates, the PHA storage yield, the maximum polymer content achievable in the biomass and the produced polymer at the end of batch accumulation test. The biomass was withdrawn from SBR at the end of the feast phase (when the content of PHA was high), put in a smaller reactor (working volume 500 mL, same temperature and pH as the second stage), diluted four times with the same mineral solution used for the SBR, with the only difference that it did not contain any nitrogen source, in order to prevent the cellular growth and to increase the achievable polymer content. Thus, the only nitrogen available at the beginning of the tests was the ammonia residual content in the mixed liquor withdrawn from the SBR. The diluted biomass was then spiked with multiple additions with the same substrates fed to the second stage in order to saturate the biomass storage capacity; the initial substrate concentration was 1000 mgCOD L^{-1}. The mixed liquor in the reactor was sampled at regular intervals for analytical determination of the substrates, PHA, and ammonia. Before (at least 20 minutes) and during the test, the batch reactor was maintained under air bubbling (at oxygen concentrations in the range 7-8 mg L^{-1}). In order to measure the oxygen uptake rate (OUR), the aeration was interrupted at intervals and the dissolved oxygen decrease was measured as a

function of time (however, oxygen concentration never decreased at concentration lower than 2 mg L^{-1}).

4A.1.3 Analytical Methods

The substrates (acetate and propionate) were measured on filtered samples (0.45-μm porosity) by gas chromatography (stationary phase Carbowax 20 M 4% on CarboPack B-DA on a Perkin-Elmer 8410 instrument). Ammonium ions were measured on filtered samples by Nessler spectrophotometric method (the absorbance of the coloured complex was measured at 410 nm wavelength; Varian DMS 90 UV-Visibile Spectrophotometer).

For PHA determination, the mixed liquor sample was treated immediately with a NaClO solution (7% of active Cl_2) and stored at -4°C for the following analysis. PHAs were extracted, hydrolysed and esterified to 3-hydroxyacyl methyl esters, and determined by gas chromatography (Braunegg et al. 1978). The relative abundance of HB and HV monomers were quantified by using a commercial polymer P(HB/HV) of known HV content, as standard (Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid), PHV content 12% wt, Sigma-Aldrich).

The non-polymer biomass (active biomass X_A) was calculated from the difference between VSS and PHA in the sample and converted into COD according to a conversion factor of 1.42 mgCOD mg^{-1} biomass: $X_A = (VSS\text{-}PHA) \bullet 1.42$. The latter conversion factor was obtained by considering the generic heterotrophic biomass formula $C_5H_7O_2N$ (Gujer and Henze, 1991).

PHAs were also converted into COD according to oxidation stoichiometry: 1.67 mgCOD mg^{-1}HB monomer, and 1.92 mgCODmg^{-1} HV monomer. The PHAs content of the biomass was calculated by dividing the measured PHA concentration by biomass concentrations (both expressed as COD).

4A.1.4 Calculations

In the SBR, the amount of stored PHA (ΔPHA) was calculated as the difference between PHA concentration in the mixed liquor at the end of feast phase (i.e. the substrate depletion time) and at the start of the respective cycle. The specific PHA production rate was calculated as the ratio of stored PHA and the length of feast

phase (t), per unit of non-polymer biomass (X_A): rPHA = ΔPHA/(t•X_A). The storage yield during the feast phase was determined as the ratio between the amount of stored PHA (as COD) and the amount of the removed substrate (as COD) fed in the cycle: Y_{STO} = ΔPHA/ΔS. The observed yield was determined as the ratio between the non-polymer biomass (X_A) concentration at substrate depletion, and the amount of substrate (as COD) fed in the cycle: Y_{OBS} = X_A/ΔS. The polymer content in the biomass was calculated (in terms of COD) at substrate depletion time as the ratio of PHA concentration and volatile suspended solid concentration (i.e. the sum of non-polymer biomass and produced polymer): %PHA=ΔPHA/VSS= ΔPHA/(X_A+ΔPHA). In batch tests the specific rates (storage and substrate consumption) were calculated by linear regression of data versus time, with reference to the first substrate addition only, in order to not include possible rate decrease due to saturation effects. For each batch test, the biomass concentration was calculated from the dilution ratio from the SBR to the batch test (1:4) and it was considered constant at its initial value, since the formation of new active biomass was practically negligible due to low growth yield and nitrogen limitation. The maximum polymer content in the biomass obtained during each test was also calculated based on PHA profiles; the storage yield was calculated only relative to the time when the maximum polymer content was achieved.

Based on results from short-term SBR runs and related batch tests, a hypothetical PHA productivity (gPHA L^{-1} d^{-1}) was calculated by considering a novel scenario where the whole biomass could be used for PHA accumulation, by feeding the SBR at a higher substrate load and harvesting the whole biomass from the SBR at the end. Hence, the PHA productivity was calculated from the final PHA concentration (g L^{-1}) achievable in the SBR (as estimated from batch test results) divided by the SBR volume (1 L) and the time passed. The latter was calculated by the sum of the time passed from SBR inoculum when the biomass was sampled and the batch test duration. The PHA productivity when using two separate reactors for II and III stages was calculated elsewhere (Beccari et al. 2010).

4A.1.5 DGGE analysis of the bacterial community during acclimation

The structure of the bacterial community was investigated throughout the biomass adaptation associated to the long SBR run lasting more than 150 days. Samples of biomass were collected from the reactor at the beginning (day 0) and after 1, 2, 7, 30, 50, 120 and 154 days of process operation and stored at -20°C in 50% (v/v) ethanol. Metagenomic DNA was extracted from approximately 250 mg of pellet with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR amplification of the bacterial 16S rRNA genes of the community was performed with primers GC-357f and 907r (Sass et al. 2001) as described elsewhere (Zanaroli et al. 2010). PCR products were resolved with a D-Code apparatus (Bio-Rad, Milan, Italy) on a 7% (w/v) polyacrylamide gel (acrylamide-N,N'-methylenebisacrylamide, 37:1) in 1× TAE with a denaturing gradient from 40% to 60% denaturant, where 100% denaturant is 7 M urea and 40% (v/v) formamide. The electrophoresis was run at 90 V for 15 h at 60 °C. The gel was stained in a solution of 1× SYBR Green I (Sigma-Aldrich, Milwaukee, WI) in 1× TAE for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy). Subsequently, a matrix of similarities between the densitometric curves of the band patterns was calculated based on the Dice coefficient with Quantity One 4.5.2 software (Bio-Rad). Finally, the DGGE patterns were clustered based on the unweighted pair–group arithmetic average (UPGAMA) clustering algorithm.

4A.2 Results

4A.2.1 SBR Runs (biomass adaptation)

4A.2.1.1 Short-term SBR Runs

Figure 4A.1B shows the length of the feast phase (substrate depletion time) of each cycle during the four short SBR Runs . As above reported, the length of feast phase corresponds to the time of substrate depletion, as easily indicated from the oxygen profile during each cycle (figure 4A.1A). In general a wave sequence of alternating short and long feast phase was observed for Runs I - III: indeed, feast phase length was changing in the range 15-120 minutes (in the latter case, being equal to the overall cycle length) with no single monotonic trend from long to short feast phase.

On the other hand, the intensity of wave pattern was decreasing with time so anyway showing a tendency to stabilize down to a shorter feast phase length. The only exception is Run IV that anyway was stopped during first peak and so before the expected new decrease of feast phase length. The length of each SBR Run was not defined in advance; it was established based on the observed profiles of the feast phase length, in order to make it possible to carry-out batch tests corresponding to high and low value of feast phase length.

Even though a general trend was observed, from figure 4A.1B it is also clear that the observed trends were different and poorly reproducible each other. The observed difference among these short SBR Runs can be likely attributed to the dynamic response of the different inocula under the imposition of strong variations of feeding conditions with respect to previous ones in the full scale plants. The poor reproducibility of the observed trends can be justified by the inevitable differences among the inocula used in the four SBR Runs. Indeed, the inocula are complex ecosystems sensitive to the fluctuations of operational factors (such as hydraulic regime, pH, temperature, wastewater, composition, etc..) that occur in activated sludge wastewater treatment plants. Consequently, the tested inocula show different abilities to adapt themselves to the strong variation of feeding conditions passing from the full scale plants to the SBR. The short duration of the runs in SBR amplified the differences in adaptation shown by the different inocula.

Even though new feeding conditions were the same for the four SBR, it can be supposed that sudden and strong variations amplified small differences among the different inocula. As an example, run III was characterized by a more stable behavior with respect to Runs I and II.

As the length of feast phase changed irregularly, the selective pressure in favor of PHA storing microorganisms varied during each SBR Run. Accordingly, figure 4A.2A shows the time profile of specific storage rate during the feast phase for the different SBR Runs. The obtained values varied in a quite wide range between 4 and 361 mgCOD gCODnonPolym^{-1} h^{-1}; one or two peaks of high storage rate appearing in each Run. Again, the time profiles of storage rate were different in each SBR Run and related peaks were also shifted between the different runs in terms of day. This confirms the poor reproducibility of process performance in the startup period, early

after each inoculation with activated sludge. Figure 4A.2B shows that the polymer content in the biomass at the end of feast phase was also widely variable during each run and from one run to the another one. As an example, in Run I the maximum polymer content appeared after 5 days from the start-up (29% COD COD^{-1}); in Run II and III, PHA peaks were very similar each other but at a quite lower value and shifted of two days before than Run I; in Run IV the maximum polymer content were obtained after 9 days from the inoculum, immediately before the run was stopped. These variations were probably due to that, under feast and famine regime, the biomass need to adapt itself to both store intracellular PHA during the feast phase and to reuse PHA during the famine phase. It can be argued that adaptation to store PHA is quicker than to reuse PHA; hence PHA are initially accumulated inside cells and this in turn can decrease the substrate uptake rate in the following cycles (Beccari et al. 2010). Considering the high performance variability observed for all four runs during the SBR start up, average values and relative standard deviation of monitored parameters were not calculated.

4A.2.1.2 Long-term SBR Run

In order to better interpret such a high variability of short-term SBR Runs, a fifth SBR Run was started and performed for a much longer period. Figure 4A.3 shows the trend of the feast phase length as a function of days passed from inoculum, during the long-term SBR Run. Even in this case, the unstable process performance immediately after the inoculum is evident. However, the frequency and intensity of oscillating length of the feast phase strongly decreased with time, likely because of the ongoing acclimation of the biomass under new dynamic feeding. An almost stable SBR performance was achieved after ~15 days (180 cycles) from the initial inoculum; the residual small fluctuations observed during the pseudo-steady state were almost totally correlated to the lack of SBR cleaning in each week-end (with formation of some biofilm on the SBR glass walls, see Material and Methods). Figure 4A.4A-B shows the specific storage rate and the polymer content in the biomass at the end of feast phase during the long-term SBR Run: the storage rate trend confirmed the high performance instability during the initial acclimation phase,

whereas much smaller fluctuations were observed for the rest of the run (even if higher values were obtained just at the end of the SBR Run).

As for PHA content in the biomass a quite high polymer content (58% COD COD^{-1}) was observed already after 3 days from the inoculum, confirming the hypothesis of a quicker adaptation to store PHA in the feast phase than to reuse PHA in the famine phase. Then, the PHA content stabilized at around 10% for the rest of the SBR Run.

Table 4A.1 summarizes average values of the measured parameters in this Run ("steady state" period from day 15 to day 154): the feast phase percentage was less than 20% of the overall cycle length; this guaranteed an adequate selective pressure on the PHA-storing microorganisms. This was confirmed by the good storage capacity during the feast phase: the specific storage rate was high (190 mgCOD $gCODnonPolym^{-1}$ h^{-1}) as well as the storage yield (0.32 COD COD^{-1}). It is worthwhile mentioning that this good storage performance was obtained during the feast phase with no nitrogen limitation.

As for microbiological analysis, remarkable changes occurred in the structure of the bacterial community throughout the run (figure 4A.5). In particular, DGGE patterns at days 0, 1 and 2 were highly similar to each other and sub-clustered together within a cluster including day 7. In addition, high similarity was observed between patterns at days 30 and 50 and patterns at days 120 and 154, which sub-clustered separately and clearly distinguished themselves from patterns at days 0-7. This indicates that the inoculum progressively evolved throughout the SBR Run. The quickest variation of microbial composition in the period between the inoculum and day 7 well reflects the higher variability of SBR performance during start up. Changes of the structure of the microbial community lasted during the "steady state" period, even though at a lower rate, but they did not cause substantial changes of PHA storage rate; e.g. very similar specific storage rates observed at days 30, 50 and 120 (~166-189 mgCOD $gCODnonPolym^{-1}$ h^{-1}). On the other hand, at day 120 the PHA storage rate was quite more similar to days 30-50 in spite that the structure of microbial community was more similar to the following day 154, when the PHA storage rate showed a new peak. At days 2 and 154 the highest storage rate values were obtained (350 and 284 mgCOD $gCODnonPolym^{-1}$ h^{-1} respectively), in spite of the very low similarity of the respective microbial communities.

These results suggest that the capability to store PHA was not strictly correlated to the structure of the bacterial community, but also that feast and famine conditions were anyway able to steadily select for PHA-storing microbial species with high PHA storage capabilities.

4A.2.2 Batch tests from SBR run (PHA accumulation)

4A.2.2.1 Effect of acclimation time to "feast and famine" regime during start-up

During each of short SBR Runs (I-IV), two or more batch tests were conducted with biomass sampled from the SBR at a pre-chosen cycle. The SBR cycles in which the biomass was sampled were characterized by a wide range of feast phase length (from 12.5% to 88.3% of overall cycle length); this means that different selective pressure were existing at the time that biomass was sampled from each SBR Run, regardless the time passed from the initial inoculum.

As an example of batch test profiles, figure 4A.6A shows the oxygen uptake rate (OUR), ammonia concentration and PHA content in the biomass in a batch test (Batch test IX) carried out sampling the biomass after 7 days from the SBR start-up (IV SBR Run) and during a cycle in which the substrate depletion time was 15 minutes (feast phase at 12.5% of the overall cycle length, corresponding to a strong selective pressure). The OUR shows a strong increase compared to the initial endogenous metabolism; after the first substrate addition, it continued to increase at a slower rate until the ammonia is depleted in the medium (more or less 2 hours). Following next additions, it tends to decrease as a consequence of lack of nitrogen in the medium which prevented new biomass emergence and also due to the gradual saturation of storage capacity. Accordingly, the substrate uptake rate tends to decrease during the test as shown by the decrease of the slope after the following additions (figure 4A.6B); the production of PHA almost entirely occurred in the first 4 hours of the test (figure 4A.6B), then it stopped likely because of exhausted biomass storage capacity as confirmed by the almost total invariance of the OUR. The specific storage rate was 330 mgCOD gCODnonPolym^{-1} h^{-1} (in the first 2 hours of the test), i.e. the biomass continued to store the polymer at the same specific rate of the feast phase of the SBR cycle (7th day of the Run IV, figure 4A.4A). The amount of produced polymer in this test was 1080 mgCOD L^{-1}, the maximum

polymer content in the biomass was 55.8% COD COD^{-1} and the corresponding storage yield was 0.45 COD COD^{-1}. These results confirmed the good storage properties of the biomass sampled from a SBR cycle that was characterized by a short feast phase This means that the biomass quickly acclimated itself to the feast and famine condition imposed in SBR.

In a batch test carried out after only 2 days from the previous one (test X, day 9^{th}, Run IV), the storage performance was significantly less. The specific storage rate was only 5.0 mgCOD gCODnonPolym$^{-1}h^{-1}$ and the amount of produced polymer at the end of the test was 55 mgCOD L^{-1}. As a consequence, the maximum polymer content was 15.8% COD COD^{-1} and the corresponding storage yield 0.02 COD COD^{-1}. Hence, after 9 days of acclimation the biomass response in the start-up period of SBR run was still unstable.

Table 4A.2 summarizes the results obtained in all batch tests carried out during the four SBR Runs in the period of start-up. These results confirm a high variability in performance that was completely unrelated to the time passed from the SBR inocula. On the contrary, a correlation between the length of feast phase in the SBR cycle in which the biomass was sampled and storage properties exhibited in the corresponding batch tests was clearly found. Regardless from the day during SBR start-up in which biomass was sampled, figure 7 shows that best storage performances were obtained when the feast phase of the biomass sampling cycle was lower (at least ≤ 20% of the overall cycle length). The best storage performances were reached in four batch tests characterized by quite short feast phase (Test III, VII, VIII, IX) that showed the highest values of specific storage rate (in the range of 182 to 330 mgCOD gCODnonPolym^{-1} h^{-1}), storage yield (0.44 – 0.49 COD COD^{-1}) and maximum polymer content (38% – 55.8% COD COD^{-1}). This confirms that a strong selective pressure on the biomass in the SBR (optimal regime of feast and famine) was necessary to obtain high storage response.

In the tests characterized by a longer feast phase of the biomass sampling cycle, the exhibited storage properties were substantially lower (i.e. in tests I, II, V and VI, when the feast phase was in the range 25% - 36.7%, too high to obtain a prevailing storage response). As a further example, Test IV and X, were conducted after 7 and 9 days from the SBR start-up respectively; hence the acclimation phase was quite

longer than batch tests III and VIII, that were carried out after 4 and 3 days from the start-up respectively. Despite the longer acclimation period, tests IV and X showed a complete loss of biomass storage properties due to very long feast phase (88.3% and 82.5% of the overall cycle length, respectively). This means that, at this high feast phase length, the selective pressure imposed to the biomass in the SBR was almost completely lost.

Overall, table 4A.2 and figure 4A.7 confirm that the storage rate and yield exhibited in the batch tests were inversely correlated to the length of feast phase in the SBR cycle in which the biomass had been sampled whereas they were completely unrelated to the time passed from the SBR inocula.

4A.2.2.2 Effect of long acclimation time (pseudo steady-state)

The biomass response in the third stage was also studied through batch tests carried out even with sludge sampling from SBR under stable operating conditions (pseudo steady-state).

The time profiles of the main parameters of accumulation tests carried out with fully acclimated biomass sampled from the long-term SBR Run (not reported) were very similar to the profiles of figure 4A.6A-B that had been obtained with quickly acclimated biomass taken in a SBR cycle with very short feast phase.

Table 3 shows the average results from these batch tests and compare them with average results from tests performed with quickly acclimated biomass sampled during SBR start-up (only tests with short feast phase conditions were selected, i.e. tests III, VII, VIII and IX). Even though the biomass samples were characterized by quite different acclimation period to the feast and famine conditions, their storage performance was very similar as for specific storage rate, storage yield and maximum polymer content reached in the biomass. The key factor, common to these obtained performances, was the short feast phase corresponding to the SBR cycle in which the biomass was sampled.

4A.3 Conclusions

This study investigated how quick is the acclimation of an activated sludge to the newly imposed feast and famine regime (in a SBR) and how it affects the storage performance of acclimated biomass.

A short feast phase length during the SBR cycle (less than 20% of the cycle length) was the key parameter to obtain good selective pressure on PHA-storing microorganisms and consequently PHA storage at high rate and yield. This condition was reached quickly, in no more than 40 cycles and no less than 22 cycles (3.33 and 2 HRTs respectively). However, once reached for the first time, the short feast phase was not maintained and a highly unstable SBR performance was observed for at least 8-10 days. In an additional long-term SBR run, a stable performance was obtained only after 15 days (15 HRT) and steadily maintained for a quite longer period.

It is worthwhile to mention that the best storage properties exhibited by quickly acclimated biomass after 22-40 cycles were very similar to those obtained with fully acclimated biomass, characterized by a longer acclimation period. This evidence suggests a new strategy for operating the overall PHA production in a single reactor instead of using two separate reactors for the biomass enrichment and the PHA accumulation, respectively. In this novel approach, the first achievement of a short feast phase in the SBR can be easily verified by on line monitoring of the dissolved oxygen profile. As soon as the short feast phase has been reached, the PHA accumulation stage is directly performed in the SBR, by feeding the biomass at a higher substrate load; finally, the whole biomass is harvested from the SBR for downstream processing (PHA extraction and purification). Based on present experimental data, the best PHA productivity was around 0.85 gPHA L^{-1} d^{-1} and final PHA content of 0.48 COD COD^{-1} basis (batch test VIII). Even though this value is lower than best PHA productivity achievable by using the usual two reactor approach (1.6 ± 0.1 gPHA L^{-1} d^{-1}, as reported elsewhere, Beccari et al. 2010), the proposed approach is anyway attractive because of its simplicity (a single reactor instead of two ones), also considering that this single reactor performance can be certainly optimized by investigating the SBR start up in a wider range of typical operating parameters (e.g. OLR, cycle length).

It is also noteworthy, that during the long-term SBR experiment similar performance was obtained even with low similarity of the structure of the microbial consortium. Cluster analysis of DGGE profiles showed that a slow drift of the microbial structure lasted for the whole long-term SBR run and so that the good storage performance and the structure of microbial community were not fully correlated. In other words, these results indicate that feast and famine conditions were anyway able to steadily select for PHA-storing microbial species with high PHA storage capabilities, in spite of small changes of microbial structure.

This evidence can be relevant because changes of microbial population could in principle affect the molecular weight (MW) and other properties of produced PHA. Hence, the short-term acclimation used in this study does not necessarily mean a larger variability of PHA properties. More research is clearly needed on this topic, also depending on PHA end-uses, towards the perspective of full exploitation of PHA production from microbial mixed cultures.

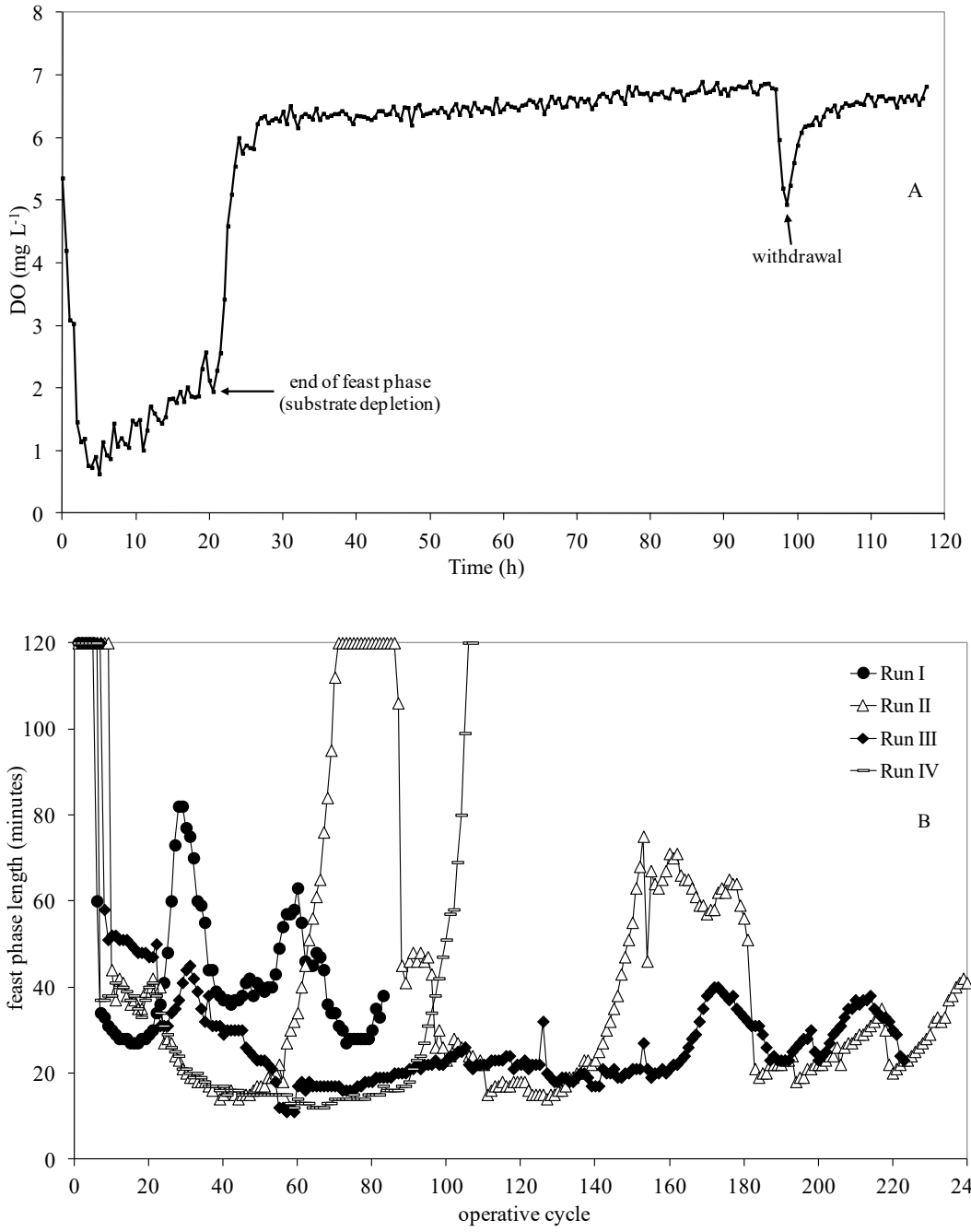


Figure 4A.1. Typical oxygen profile from which the length of feast phase was calculated (1-A) and time profiles of length of the feast phase during the four short SBR runs (1-B)

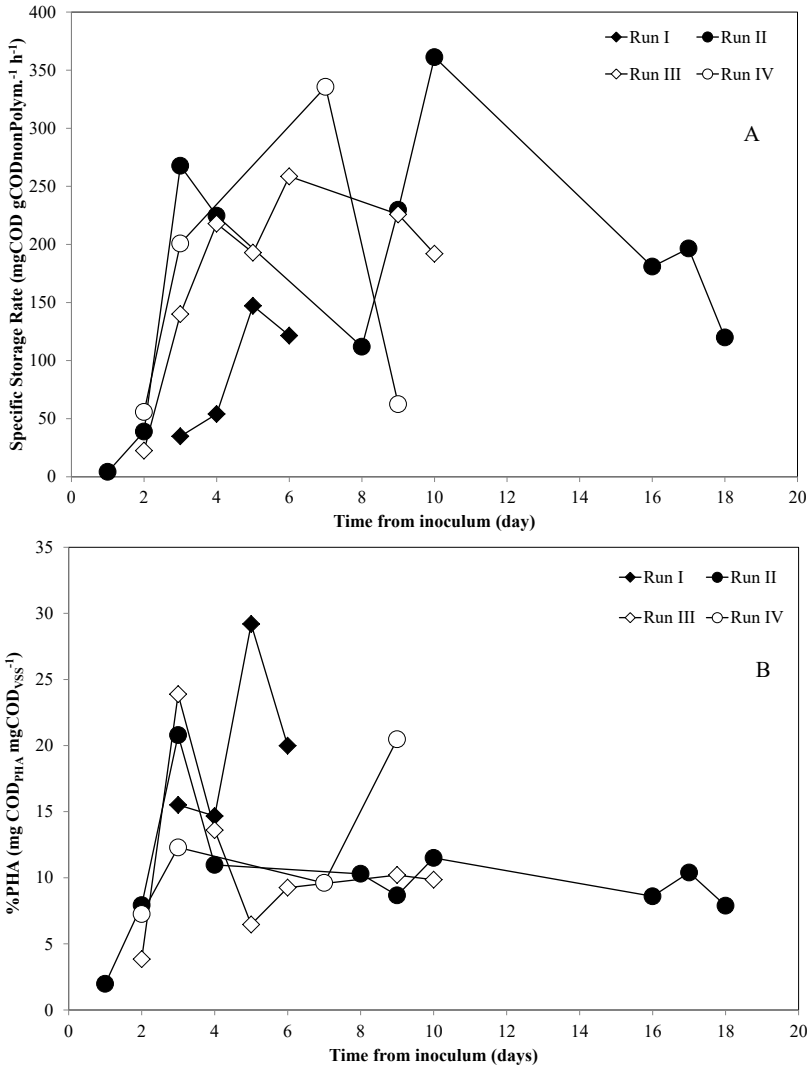


Figure 4A.2. Specific Storage Rate (A) and Polymer content in the biomass (B) at the end of feast phase in all short SBR runs during the start-up

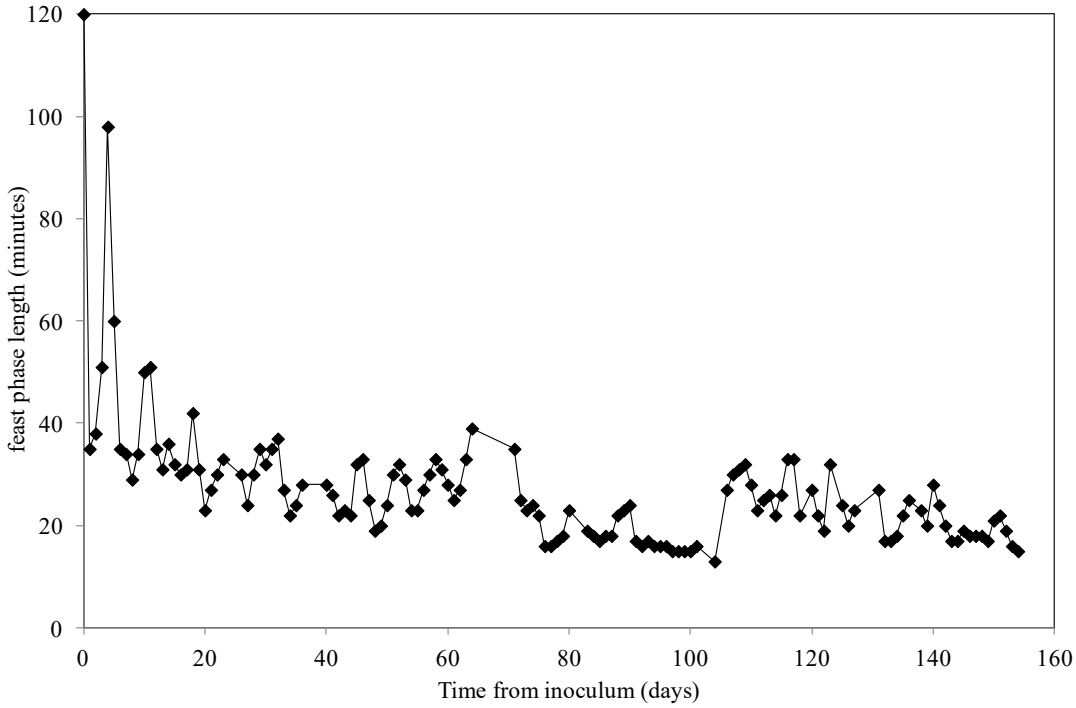


Figure 4A.3. Length of the feast phase during the long SBR run

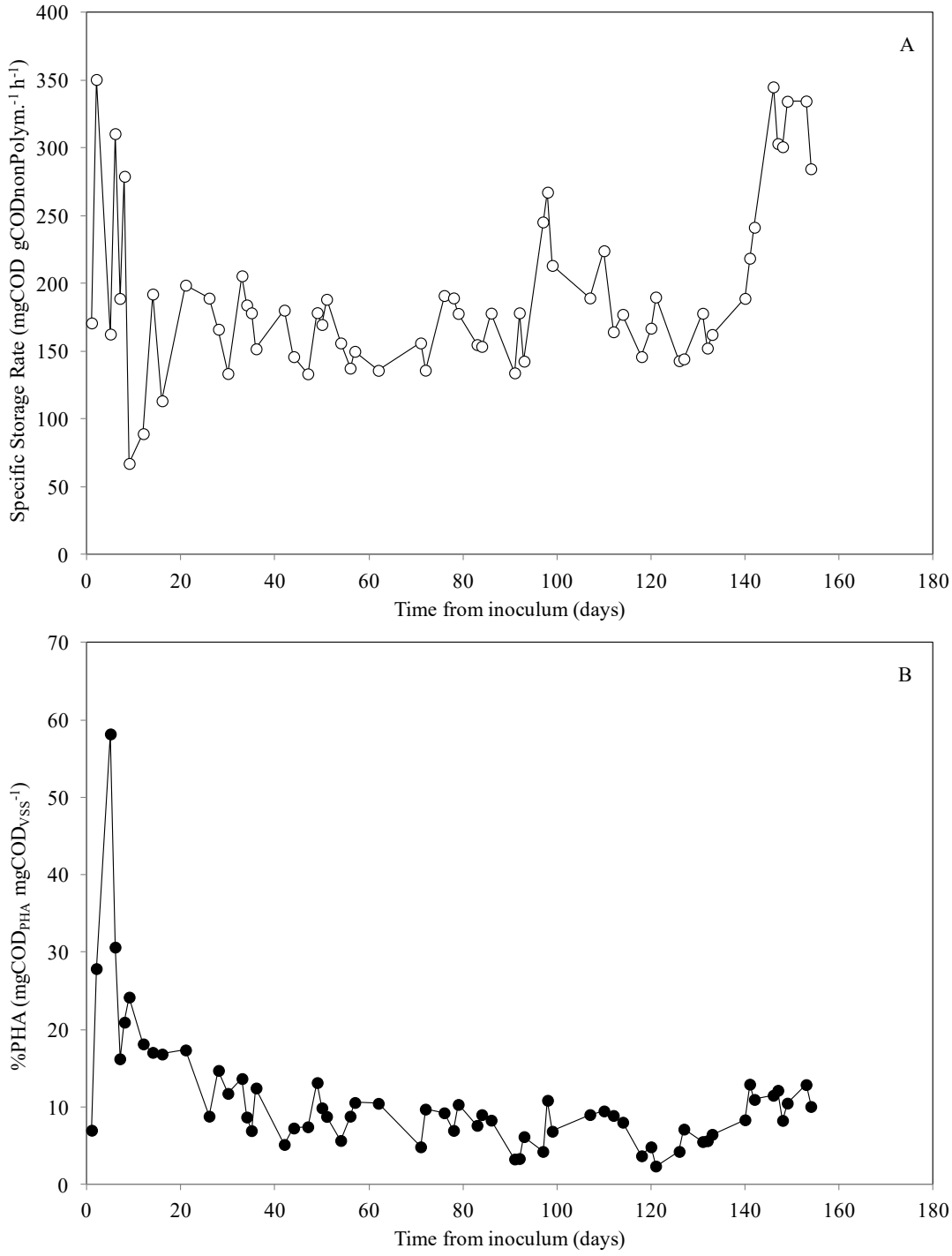


Figure 4A.4. Specific Storage Rate (A) and Polymer content in the biomass (B) at the end of feast phase during the long SBR run

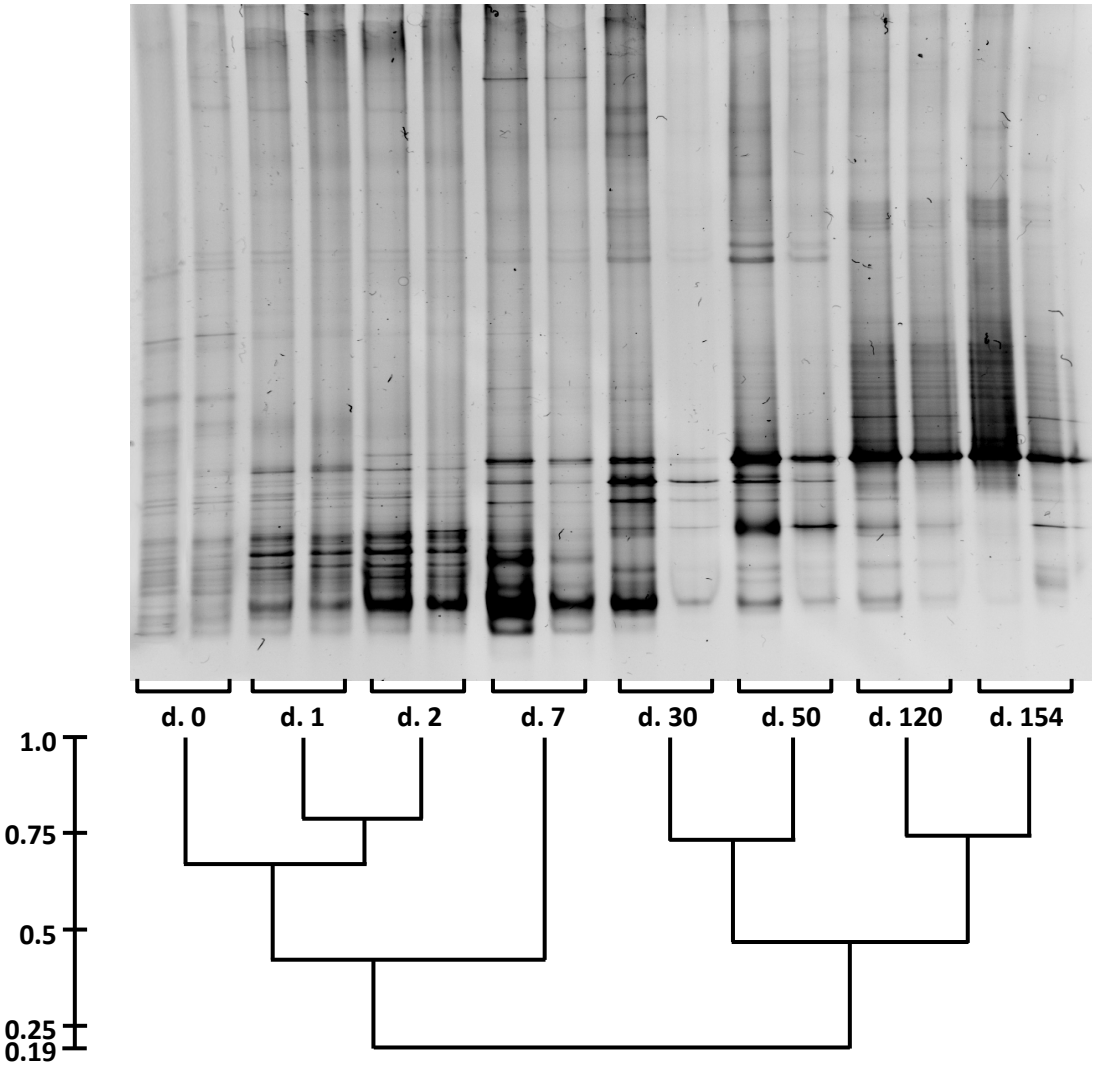


Figure 4A.5. Structure of the bacterial community throughout the long term SBR run. DGGE patterns were clustered with the unweighted pair-group arithmetic average (UPGAMA) clustering algorithm

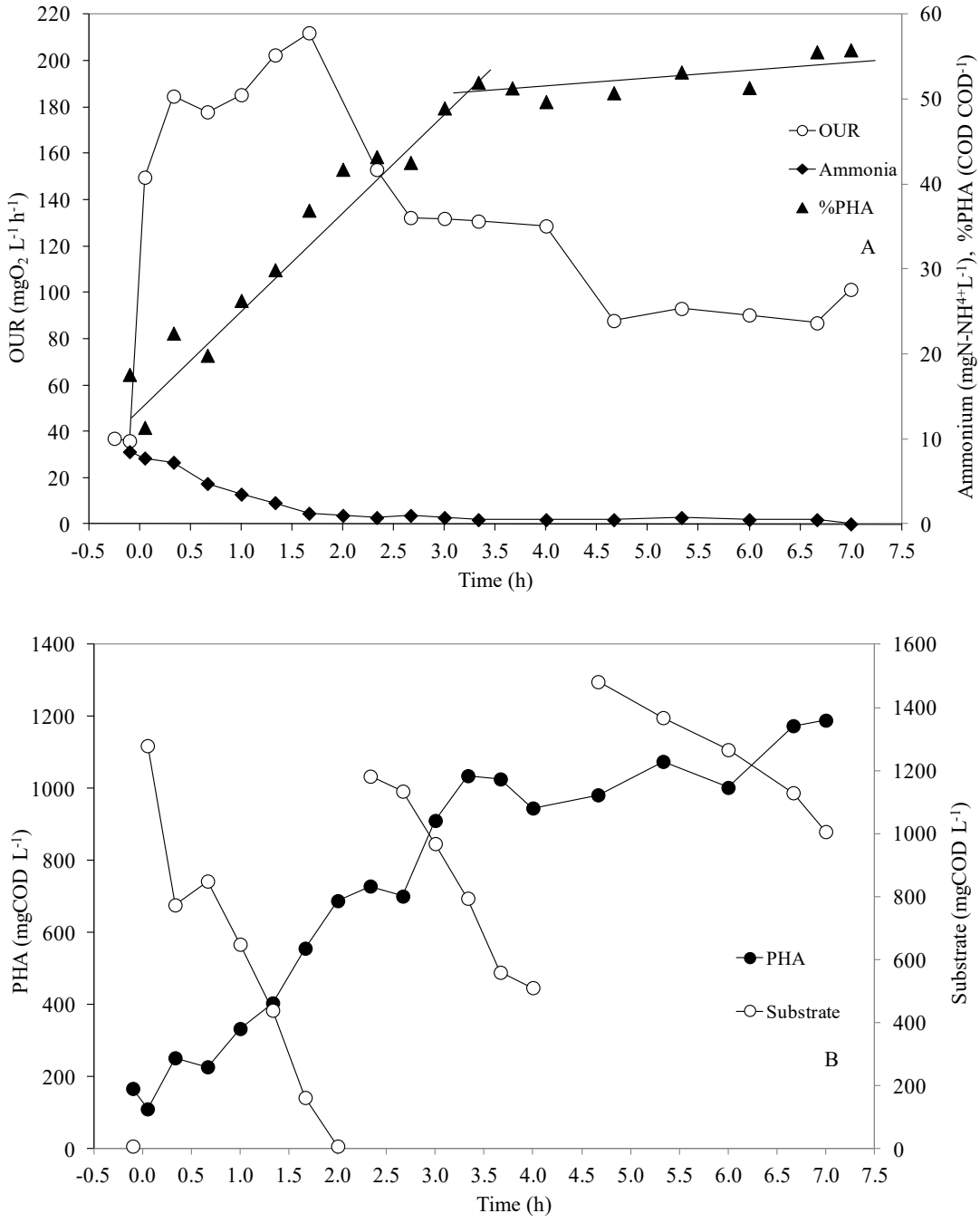


Figure 4A.6. OUR, Ammonia concentration and %PHA (A), Substrate and PHA concentration (B) in a batch test carried out with quickly acclimated biomass (run IV, batch test IX, biomass taken from a SBR cycle with very short feast phase)

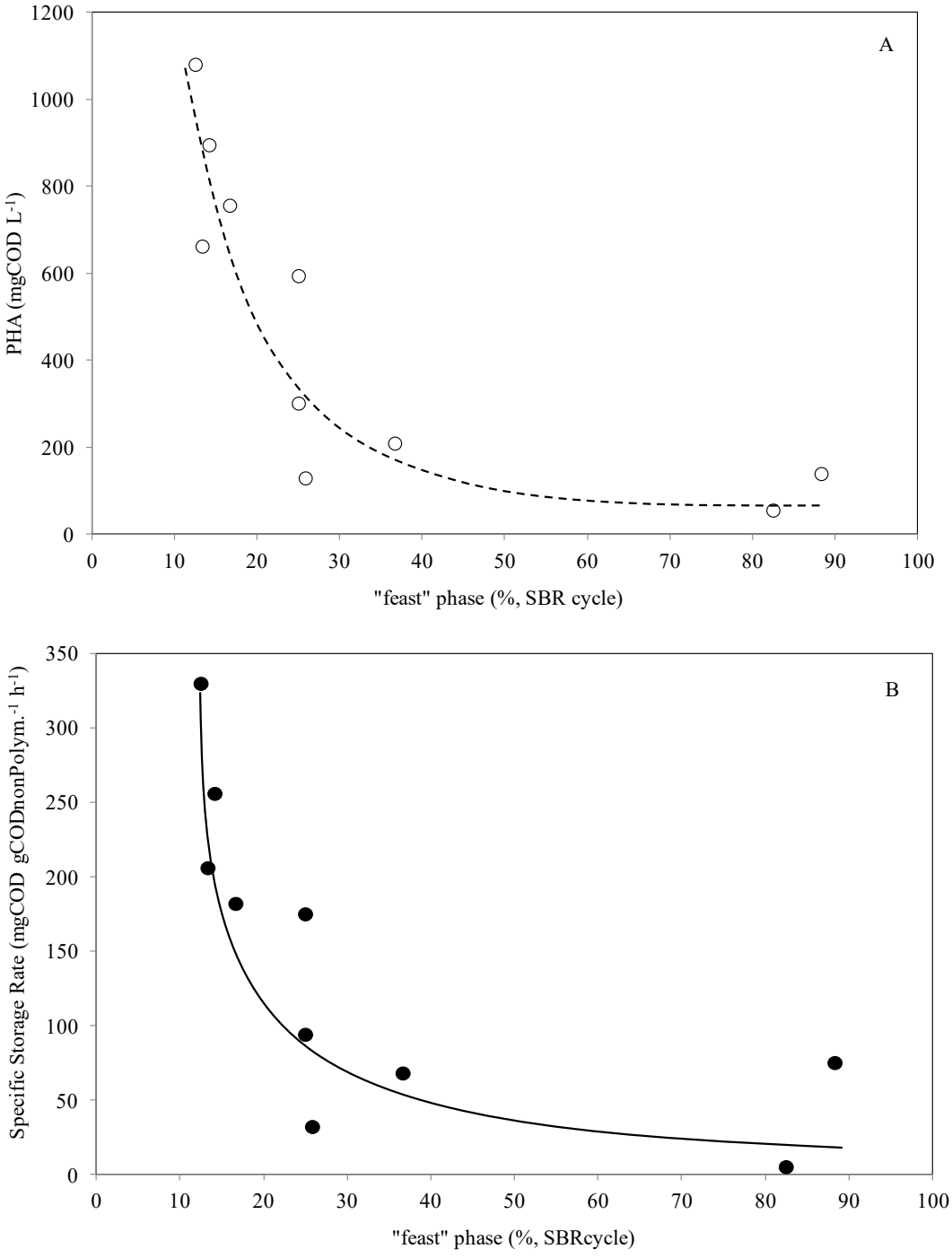


Figure 4A.7. Produced Polymer (A) and Specific Storage Rate (B) in batch tests as function of feast phase of the SBR cycle from which the biomass was sampled

Parameter (SBR Run, OLR 8.5gCOD L^{-1} d^{-1}, HRT 1day, overall cycle length 2 h)	
nonPolymer Biomass concentration at substrate depletion (mgCOD L^{-1})	3098 ± 104
PHA concentration at the end of cycle (mgCOD L^{-1})	124 ± 7
PHA concentration at substrate depletion (mgCOD L^{-1})	374 ± 13
feast phase length (min.)	23.6 ± 0.6
feast/famine ratio (%)	19.7 ± 0.5
specific substrate removal rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	612 ± 26
specific storage rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	190 ± 7
Observed Yield (Y_{OBS}^{SBR}; COD COD^{-1})	0.40 ± 0.01
Storage Yield (Y_{STO}^{feast}; COD COD^{-1})	0.32 ± 0.01

Table 4A.1. Average values during pseudo-steady state of measured parameters in long term SBR run

Partially acclimated biomass										
Parameter	Run I		Run II			Run III		Run IV		
	Test I	Test II	Test III	Test IV	Test V	Test VI	Test VII	Test VIII	Test IX	Test X
Day of the SBR run (from the inoculum)	3	6	4	7	8	2	5	3	7	9
Biomass sampling cycle	37	72	49	87	98	23	62	33	82	105
feast phase length (biomass sampling cycle, min.)	44	30	17	106	30	31	16	20	15	99
feast/famine ratio (biomass sampling cycle, %)	36.7	25	14.2	88.3	25	25.8	13.3	16.7	12.5	82.5
Specific storage rate (rPHA, $mgCOD_{gCODnonPolym}^{-1}\ h^{-1}$)	68	175	256	75	94	32	206	182	330	5
Maximum polymer content (%, $COD_{PHA}\ COD_{VSS}^{-1}$)	22.1	41.4	48.7	25.6	32.3	14.8	38	48.4	55.8	15.8
Storage Yield (Y_{STO}, $COD_{PHA}\ COD_{gS}^{-1}$)	0.09	0.23	0.44	0.26	0.18	0.1	0.49	0.48	0.45	0.02
Produced PHA ($mgCOD L^{-1}$)	209	594	895	139	301	129	662	756	1080	55
Final PHA concentration ($mgCOD L^{-1}$)	379	753	1061	267	455	189	734	866	1190	177
Initial nonPolymer biomass concentration (Xa, $mgCOD L^{-1}$)	841	887	863	625	890	1083	1088	785	854	637

Table 4A.2. Summary of batch tests carried out during the different short SBR runs in unstable operating conditions

	Quickly acclimated biomass (SBR start-up)		**Fully acclimated biomass (SBR pseudo-steady state)**	
Parameters	range	mean value	range	mean value
Day of the SBR Run (from the inoculum)	3 - 7	4.8 ± 0.9	146 - 153	150 ± 4
Biomass sampling cycle	33 - 82	57 ± 10	1746 - 1830	1788 ± 42
feast phase length (biomass sampling cycle, min.)	15 - 20	17 ± 1	18 - 16	17 ± 1
feast/famine ratio (biomass sampling cycle, %)	12.5 - 16.7	14.2 ± 0.9	15 - 13.3	14.2 ± 0.8
specific storage rate ($mgCOD\ gCODnonPolym.^{-1}\ h^{-1}$)	182 - 330	244 ± 33	183 - 209	196 ± 13
Maximum polymer content (%, $COD_{PHA}\ COD_{VSS}^{-1}$)	38 - 55.8	48 ± 4	49.6 - 50.9	50.3 ± 0.7
Storage Yield (Y_{STO}, $COD_{PHA}\ COD_{\Delta S}^{-1}$)	0.44 - 0.49	0.47 ± 0.01	0.52 - 0.53	0.53 ± 0.01
Produced PHA ($mgCOD\ L^{-1}$)	662 - 1080	848 ± 91	852 - 897	875 ± 23
Initial nonPolymer biomass concentration (X_A, $mgCOD\ L^{-1}$)	785 ± 1088	897 ± 66	588 ± 603	595 ± 7

Table 4A.3. Range and mean values of the parameters calculated from batch tests carried-out with quickly acclimated biomass (SBR star-up) and fully acclimated biomass (SBR pseudo steady state)

4B. Effect of the cycle length on a PHA production in SBR

In establishing the selection in favor of a biomass with high storage rates through the alternation of feast and famine conditions, the organic loading rate (OLR) plays an important role. By varying the OLR at the same length of the cycle and hydraulic retention time (HRT), the amount of substrate fed per cycle varies and the length of the feast and famine phases varies as well. Most reported studies (Beccari et al. 1998; Beun et al. 2000; Beun et al. 2002; Martin et al. 2003; Serafim et al. 2004) focused on storage response of mixed cultures were carried out in the lower OLR range (approximately 0.3 - 1.2 gCOD L^{-1} d^{-1}). Under such OLR range, the feast and famine conditions were always established and the developed biomass exhibited a good storage response. The effect of higher OLR on the selection of biomass and on its storage response with high storage rates has been specifically studied in a previous study (Dionisi et al. 2006). It has been shown that the OLR affected biomass selection; at OLR equal to or less than 12.75 gCOD L^{-1} d^{-1}, the selected biomass showed a transient response that was mainly due to the storage, whereas at OLR equal to or higher than 25 gCOD L^{-1} d^{-1} biomass response was mainly due to the growth, with little or no contribution of storage. At the intermediate OLR of 20 gCOD L^{-1} d^{-1}, biomass selection was unstable, with a shift from storage to growth response during reactor operation. This means that at around 20 gCOD L^{-1} d^{-1} the extent of feast and famine conditions was not enough to select microorganisms with a storage response.

In a more recent study (Beccari et al. 2010), the effect of OLR on biomass storage response was investigated by varying the length of the cycle (and consequently the HRT) maintaining the same amount of substrate fed per cycle. The investigated OLR range was 8.5 – 40.8 gCOD L^{-1} d^{-1}, higher than previous study (Dionisi et al. 2006) and the overall cycle length was decreased from 2 to 0.42 hours. The best observed storage response was observed at the lowest OLR investigated (8.5 gCOD L^{-1} d^{-1}). However it was noteworthy that a clear and stable storage response was maintained at least up to 25.5 gCOD L^{-1} d^{-1}. This is the highest OLR never reported where mixed cultures still maintained a storage response under dynamic feeding.

On the other hand, at a fixed OLR and HRT the amount of substrate fed per cycle can be also modified by varying the length of the cycle. As a result, the frequency of

the alternation of the feast and famine phases also changes. As storage is triggered by the alternation of feast and famine conditions, it is clear that by varying the length of the cycle the relevance of the storage in the dynamic response of the biomass can be varied. A previous study (Dionisi et al. 2007) showed the relevant role of the length of the cycle on storage response of the selected biomass at fixed OLR (20 gCOD L^{-1} d^{-1}). A high storage response was obtained at intermediate values of the length of the cycle, 2 and 4 h. At low or high length of the cycle, storage rate and yields were very low and the dynamic response of the biomass to substrate excess was dominated by growth. However, in the best operating condition, the process was unstable. Thus, at lower OLR of 8.5 gCOD L^{-1} d^{-1}, fixed for all the SBR runs, the effect of the overall cycle length was investigated in the range 2-8 h; in the latter case the feeding time was changed in order to evaluate any eventual effect of the maximal substrate concentration at which the biomass was exposed at fixed feeding frequency.

In a perspective of a complete process implementation (at least for both aerobic stages of selection and PHA accumulation), the effect of an additional starvation time imposed to the biomass, acclimated in SBR operating with a cycle length of 2 h, was investigated in a short-term batch tests.

A molecular analysis of the microbial composition of the mixed culture which developed in a long-term SBR run was also carried out by means of denaturing gradient gel electrophoresis (DGGE) and PCR amplification of the bacterial 16S rRNA genes. This was done in order to evaluate any changes occurring in the structure of the bacterial community throughout the different SBR runs.

4B.1 Materials and methods

4B.1.1 SBR operation

A SBR (working volume 1 L) was utilized for culturing the activated sludge under periodic feeding conditions. The SBR was inoculated with activated sludge from the "Roma Nord" full scale plant. The experimental work was divided into three runs, conducted at the same process condition except for the operative cycle lengths: Run I (overall cycle length 8 h), Run II (6 h), Run III (2 h). Each run was operated for a long period in the range 95 – 180 days; this period appeared to be much higher than necessary to achieve a stable operating conditions (more or less 10 days).

Run I was divided in two ones: run I-a was operated until 69th day from initial inoculum; Run I-b until the end (95th day). Runs I-a, II and III were started with a new inoculum from the full scale plant (Roma Nord wastewater treatment plant). All the runs had the same length of the feed phase (10 min) except for Run I-a that was characterized by a feeding time of 40 minutes.

The biomass withdrawal was set for 1 minute; different from typical SBR operation [44], no settling phase was performed, and all excess biomass was withdrawn with the mixed liquor. In this way, SRT was set equal to HRT (1 day). The SBR feed was a mixture of acetic and propionic acid at an overall concentration of 8.5 gCOD L^{-1} with a resulting OLR of 8.5 gCOD L^{-1} d^{-1}. The relative amounts of acetic and propionic acid were 85% and 15% on a COD basis. The SBR feed also contained the mineral medium (composition in paragraph 3A.1.1). The system was set at the same operating conditions already described in 4A.1.1, as well for sampling activity.

During each SBR Run, it was considered that a pseudo-steady state was obtained when feast phase length remained approximately constant (within 5% deviation from average values) for at least 5 consecutive days.

4B.1.2 Batch accumulation tests

Batch tests were carried out in a 500 mL working volume reactor at the same temperature and pH as the SBR, after a pseudo-steady state in the SBR was reached.

The first experimental tests batch set was conducted during the SBR runs to verify if the biomass dynamic response was dependent on the previous cultivation conditions, by sampling the biomass from SBR at substrate depletion. The biomass was withdrawn from SBR and diluted to a lower concentration, in the range 600 – 1200 mgCOD L^{-1} (the wide range was due to the quite different biomass concentration in SBR), with the same mineral solution used for the SBR, but without $(NH_4)_2SO_4$ (see paragraph 4A.1.2); the initial ammonia concentration in batch test was in the range 10-20 mg L^{-1}, usually depleted in the first hour or in the first two hours of the tests. The method used to conduct the tests was explained in the paragraph 4A.1.2.

During the Run III, a second experimental set of batch tests (short-term batch tests) was also conducted, in order to investigate the effect of starvation on the specific storage rate and yield of selected biomass, by sampling the biomass from the SBR at

different times: substrates depletion time (no starvation), end of the cycle, end of the cycle with additional two hours of starvation; in which the biomass was maintained under air bubbling without substrate; end of the cycle with four additional hours of starvation. The biomass was withdrawn from SBR and diluted to a lower concentration, in the range 600 – 800 mgCOD L^{-1}, with the same mineral solution used for the SBR, but with a lower amount of $(NH_4)_2SO_4$, in order to make the ammonia concentration more easily measureable. Then, biomass was spiked with a mixture of the same substrate already present in the feed at an overall concentration of about 700 mgCOD L^{-1}, in order to obtain approximately an initial S/X ratio equal to 1.0 mgCOD $mgCOD^{-1}$, contributing to the total substrate uptake in the tests.

4B.1.3 Analytical methods

See paragraph 4A.1.3

4B.1.4 Calculations

In the SBR, all the parameters were calculated as described in the paragraph 4A.1.4, as well for long-term batch tests under nitrogen limitation.

In batch tests with higher initial nitrogen concentration (short-term), the specific storage and substrate uptake rates were calculated with reference to the first two hours of each test, by linear regression of experimental data. Differently from the nitrogen limitation tests described above, the grown biomass at the end of the tests was considered.

4B.1.5 Molecular analysis of microbial communities

The evolution of the bacterial community structure and composition during SBR runs was investigated via PCR-DGGE analysis of 16S rRNA genes. Metagenomic DNA was extracted in duplicate from approximately 250 mg of sludge pellet collected at each sampling time with the UltraClean Soil DNA kit (MoBio Laboratories, Carlsbad, CA, USA), according to the manufacturer's instructions. Duplicate metagenomic DNA samples were subjected independently to PCR amplification of 16S rRNA genes and obtained amplicons loaded into adjacent lanes of denaturing gradient gels for DGGE analysis. PCR amplification of the V3-V5 variable regions

of the bacterial 16S rRNA gene was performed with primers GC-357f and 907r (Sass et al. 2001) as described previously (Zanaroli et al. 2010). PCR products were resolved with a D-Code Universal Mutation Detection System (Bio-Rad, Milan, Italy) on a 7% (w/v) polyacrylamide gel (acrylamide-N,N'-methylenebisacrylamide, 37:1) in 1× TAE with a denaturing gradient from 40% (top) to 60% (bottom) denaturant, where 100% denaturant is 7M urea and 40% (v/v) formamide. The electrophoresis was run at 55 V for 16 h at 60°C. The gel was stained in a solution of 1× SYBR Green I (Sigma-Aldrich, Milan, Italy) in 1× TAE for 30 min and its image captured in UV transillumination with a digital camera supported by a Gel Doc apparatus (Bio-Rad, Milan, Italy). DGGE bands were cut from the gel with a sterile scalpel and DNA eluted in 50 µL of sterile deionized water for 16 h at 4°C. Two µL of the eluted band fragments were then PCR re-amplified as above with non GC-clamped primers and the obtained amplicons purified with ExoSAP-IT® (Affymetrix, Santa Clara, CA) and sequenced with primer 357f. Sequencing reactions and runs were performed by BMR Genomics (Padova, Italy). The closest relative and the closest cultured relative to each phylotype were retrieved from the Ribosomal Database Project (RDP, release 10) website (http://rdp.cme.msu.edu) with the Seqmatch tool.

4B.2 Results

4B.2.1 SBR performance

4B.2.1.1 Selection of biomass under different process conditions

The SBR was firstly operated to an overall cycle length of 8 h (Run I). During Run I, the feeding time was first taken at 40 min (Run I-a) and then decreased to 10 min. (Run I-b). In following two runs, the SBR was operated to an overall cycle length of 6 h (Run II) and 2 h (Run III), while maintaining the feeding time of 10 min.

Because the daily organic load rate (OLR) and the hydraulic residence time (HRT) were left unchanged for all Runs, the variations of the frequency of feeding and of its duration as well were expected to have a strong impact on substrate profiles in the SBR. As a confirmation, figure 4B.1 shows typical oxygen profiles during a cycle for the different Runs, where the sharp drops of dissolved oxygen indicate the start of feeding and their sharp increases indicate that the given substrate has been depleted.

In other words, the dissolved oxygen profile allows to easily calculate for each cycle both the substrate depletion time (i.e. the "feast phase" length during each cycle) and the substrate removal rate (being known the amount of fed substrate per cycle).

Based on this calculations, figure 4B.2 shows the time profile during each SBR Run of the daily average of the length of feast phase. In Run I-a (40 min feed out of 8 h cycle length), there were strong fluctuations and pseudo steady-state with short feast phase was never maintained for more than 10-12 days. In Run I-b (10 min feed out of the same 8 h cycle length), a steady state condition was quickly established and maintained for at least 20 days. By maintaining the very short feed (10 min), a stable SBR performance was also established in Run II and Run III (6 and 2 h cycle length, respectively). Once a stable operating condition was established after strong initial fluctuations, steady state operation was maintained for most duration of the two Runs, even though transient periods of instability were anyway observed, lasting between 5 and 8 days. These instability periods were characterized by an increase in feast phase, sometimes up to 90% of the overall cycle length (Run I). Based on above reported profiles, transient fluctuations were individuated and excluded from the calculation of steady-state average values (table 4B.1). Dealing first with the substrate removal, table 4B.1 indicates that the length of feast phase was strongly affected by both the cycle length and the feed length. As for the effect of feed length (8 h as cycle length, Runs I-a and I-b), the feast phase length decreased from 157 to 125 min when the feed length decreased from 40 to 10 (33 and 26% of overall cycle length, respectively). A much stronger effect was observed as the cycle length decreased from 8 to 6 and to 2 h (being the feed length constant at 10 min), that caused the feast phase length decreasing from 125 min to about 74 and 24 min at 6 and 2 h, respectively (from 26 to 20.4 and 19.7%, respectively).

The latter effect on feast phase length was partially due to the change of organic load given per cycles (that increased as the cycle length increased) and partially due to the different substrate removal rate. The latter was quite higher and similar at 2 and 6 h cycles (Runs II and III) than at 8 h cycle (Run I-b).

To better describe this effect, figure 4B.3 shows the "average" substrate profiles that were calculated for each SBR Run by considering their respective feeding flow rate (L h^{-1}) and volumetric substrate uptake rate (mgCOD L^{-1} h^{-1}), the latter under the

reasonable assumption that it was almost constant for the whole feast phase, from the feeding start to the substrate depletion (see also OUR profiles in figure 4B.1). Because of different feeding time, the peak of Run I-a was lower than Run I-b (1.993 and 2.595 mgCOD L^{-1} respectively) and it took time to be reached. The latter also reflected into the above reported longer feast phase, 157 vs 125 min on average (26% and 33% of the overall cycle length, respectively). It has been widely reported (Dionisi et al. 2005b; Dionisi et al. 2006; Dionisi et al. 2007; Albuquerque et al. 2010; Beccari et al. 2010) that feast phase higher than 25% of the overall cycle length has negative effect on the selection of biomass with high storage response. Accordingly, the specific storage rate and the storage yield during the feast phase were higher in Run I-b (72 mgCOD $gCOD^{-1}$ L^{-1} and 0.23 COD COD^{-1}) than in Run I-a (19 mgCOD $gCOD^{-1}$ L^{-1} and 0.05 COD COD^{-1}). It is really noteworthy that a so dramatic impact on storage properties is caused by a very small change of operating conditions, i.e. by decreasing the feed length from about 8 to 2% of the overall cycle length, that in turn cause minor changes of the peak concentration (30 % variation) and feast phase length (26% variation).

Moreover, the biomass acclimated under Run II operating conditions (cycle length 6 h) had more similar behavior to Run III than to Runs I-a, in spite its peak profile was quite more similar to Runs I than to Run III. As an example, the peak of maximal substrate concentration obviously appeared at the same time in Run I-b, II and III (10 min feed), even if it reached quite different values: 2.595, 1.814 and 374 mgCOD L^{-1}, respectively. However, peak height of Run II was quite more similar to the Run I-b than to Run III; hence, the maximal concentration of VFAs at the end of feed does not seem to play a relevant role (at least, at this high organic load). The same rationale applies to the frequency of feed (6 h vs either 8 or 2 h, respectively) and to the length of famine phase (74 vs either 125 or 23.6 min, respectively). On the contrary, the average ratio of feast phase length relative to the overall cycle length was very similar between Run III (19.7%) and Run II (20.4%) whereas it was significantly longer in Run I-b (26%). Accordingly, both the specific substrate removal rate and the specific storage rate in the feast phase were high and similar in Run II and III (Table 1) with respect to Run I-b in which the feast phase/cycle length ratio was longer. The storage yield followed the same qualitative trend of the specific

storage rate (figure 4B.4). The same rationale also applies to explain the worst specific storage rate of Run I-a with respect to Run I-b, whose peak profiles were quite similar, but for the ratio of feast phase length relative to the overall cycle length (33 vs 26%).

In addition, the ratio between feeding time and cycle length ratio was the same in Run I-a (40 min out of 8 h) and Run III (10 min out of 2 h). However, the process performance was completely different, confirming the most relevant role of the ratio of feast phase length relative to the overall cycle length.

4B.2.1.2 Microbial communities in SBR

The structure and the composition of the microbial communities occurring in the SBR during the three runs were characterized by means of PCR-DGGE analysis of 16S rRNA genes in order to investigate how and to which extent the overall cycle length and the feeding time affect the selection of PHA-storing bacteria.

The most remarkable changes in the microbial community occurred up to day 60 in Run I, day 10 in Run II and day 30 in Run III (figure 4B.5).

A *Firmicutes* phylotype closely related to a *Planococcus* sp. (band 8-I), the Alphaproteobacterium *Paracoccus denitrificans* (Band 9-I) and a Betaproteobacterium closely related to the *Thauera* genus (band 11-I) were the dominant members in the very early stages of Run I-a, while two *Alcaligenes faecalis* strains (bands 4-I and 6-I) were dominant at day 18 and an Alphaproteobacterium closely related to *Meganema perideroedes* (band 10-I) was prominent further, although growth of *Thauera*-related phylotype was also recovered. Shortening of the feeding time from 40 to 10 minutes (Run I-b) induced the progressive loss of *Meganema perideroedes*, the enrichment of a *Hydrogenophaga* sp. strain (band 5-I) and a transient inhibition of the *Thauera* phylotype, that was not detected at day 79 while represented the most prominent community member on day 94. Indeed, the capacity of *Thauera* spp. (Serafim et al. 2006; Lemos et al. 2008), *Alcaligenes faecalis* (Schroll et al. 2001), *Meganema perideroedes* (Kragelund et al. 2005), to store PHA has been previously reported, and the presence of *Thauera* and *Meganema* has been observed in SBR for PHA production (Dionisi et al. 2005b; Dionisi et al. 2006; Beccari et al. 2009). However,

the higher transient instability of Runs I-a and I-b as compared top Runs II and III (figure 4B.2) was reflected by frequent changes in the composition of the microbial community growing in the SBR and the sequential enrichment of different bacteria with potentially different PHA storing capabilities, that probably lead to low average PHA specific storage rates and yields; this might have been the consequence of the higher maximum substrate concentration reached in these Runs (2.595 and 1.993 mgCOD L^{-1}) and thus of the wider substrate concentration range which the microbial community was exposed to during each cycle.

Conversely, at the lower cycle lengths set during Run II and Run III, corresponding to lower maximum substrate concentrations achieved at each cycle (1.814 and 0.374 gCOD L^{-1}, respectively), a more stable microbial community was enriched in shorter times after the reactor inoculation. In particular, *Hydrogenophaga flava* (band 4-II), a *Desulfibacter* sp. strain (band 5-II) and *Xantobacter autotrophicus* (band 6-II) that prevailed during the first three days of Run II were rapidly outcompeted by *Lampropedia hyalina* (band 2-II), which represented the major phylotype growing during Run II from day 10 on. Similarly, *Thauera selenatis* (band 14-III), along with some phylotypes with unknown PHA storage capabilities of the *Acidovorax* and *Dechloromonas* genera (bands 10-III, 11-III and 13-III), represented the major phylotype up to day 30 of Run III, when *Lampropedia hyalina* (band 4-III) became the dominant microorganism in the SBR. Therefore, *Lampropedia hyalina* was stably enriched in both runs where the highest average PHA storage rates and yields were observed, thus indicating that it was the main PHA storing microorganism of the community. As a matter of fact, *Lampropedia hyalina* has been recently detected as the dominant microorganisms in PHA producing SBRs (Beccari et al. 2009; Beccari et al. 2010). In addition, the highest specific substrate removal rates detected in Runs II and III, where lower maximum substrate concentrations were fed at each cycle, suggest that this microorganism was enriched under these conditions probably due to its higher affinity for the substrate.

4B.2.2 Third stage performance

The third stage of the process is necessary to increase the polymer content in the biomass by feeding again the biomass with the same types of substrates and letting

the biomass to store the substrates at its maximal content. Therefore, the performance of the third stage of the process was investigated as function of the previous biomass acclimation in the SBR runs and other operating conditions (starvation).

4B.2.2.1 Batch tests: effect of the SBR condition

The biomass response in the third stage was studied through batch tests carried out with biomass taken from the SBR during each one of the runs.

The trends of the main parameters of a typical batch accumulation test carried out with biomass sampled from SBR Run II are reported in figure 4B.6 (A-B). After the first spike, the OUR quickly increased and acetic and propionic acids were simultaneously consumed. Substrate consumption occurred along with consumption of residual ammonia and formation of PHA, indicating that biomass growth and PHA storage were occurring simultaneously. Substrate uptake rates clearly decreased after the second spike, probably due to the ammonia depletion that occurred almost totally in the first hour of the test. Correspondingly, a strong decrease of OUR was also observed, that remained almost at a constant value until the end of the test (100 mgO_2 L^{-1} h^{-1}). As expected, the storage remained active also in the absence of ammonia but it anyway occurred at lower specific rate than when ammonia uptake allowed for simultaneous growth. At the end of the accumulation test, the polymer concentration was 938 mgCOD L^{-1}, corresponding to 50.8 COD COD^{-1} as polymer content in the biomass.

Table 4B.3 summarizes the monitored parameters (with main values and standard deviation) characterizing the biomass response in the batch tests under partial nitrogen limitation. The initial specific storage rate and specific substrate uptake rates were quite similar for the biomass acclimated in Run III and II (196 and 216 mgCOD $gCOD^{-1}$ h^{-1} for the storage rate and 787 and 624 mgCOD $gCOD^{-1}$ h^{-1} for the substrate uptake rate, respectively). Both specific storage rate and substrate uptake rate were lower in batch tests conducted with biomass from Run I-b. The same considerations can be made for the maximum polymer content in the biomass, that was ~50% COD COD^{-1} in Run III and II and ~38% COD COD^{-1} in Run I-b; the highest storage yield and the highest amount of polymer produced at the end of the

tests were obtained during Run III (0.53 COD COD^{-1} and 895 mgCOD L^{-1} for the storage yield and produced polymer respectively). These results confirmed the good storage response of biomass selected in SBR operating at an overall cycle length of 2 and 6 hours (Run III and II); a further increase of the operative cycle (i.e. a decrease of feeding frequency) maintaining the same feeding time involved in a partial loss of the selective pressure and eventually a lower storage response in the accumulation tests.

The results obtained from batch tests carried-out with biomass taken from Run I-a confirmed what observed in SBR. The initial specific storage rate (34 mgCOD $gCOD^{-1}$ L^{-1}) was more than 3 times lower than value obtained in Run I-b, and almost 6 times lower than Run III. The storage yield was only 0.06 COD COD^{-1}, decreased by a factor of 10. The low storage response of the biomass in these tests, led to a maximum PHA content of 14% COD COD^{-1} only.

4B.2.2.2 Batch tests: effect of the starvation

The effect of the starvation was exploited in short-term batch tests. The biomass was taken from SBR during the Run III, at different time passed from substrate depletion and left under aeration (up to 4 h) until the new substrate spike. This strategy was made in order to investigate the instantaneous biomass response (versus growth or storage) as a function of different starvation time.

Figure 4B.7 (A-B) shows the biomass behavior (in terms of OUR and PHA trends) in different accumulation tests as function of the moment when the biomass was sampled from the SBR (either immediately after substrate depletion or at the end of the cycle) and of an additional starvation time that was imposed from the sampling time and the start of the accumulation test. The OUR profiles (figure 4B.7-A) showed that by sampling the biomass from SBR at the end of feast phase (i.e. no subsequent famine) or at the end of cycle (i.e. after the usual famine period when the biomass gets ready for the next feeding), a similar biomass response was obtained. The initial specific storage rate was very similar: 190 mgCOD $gCOD^{-1}$ h^{-1} in the test conducted with biomass sampled at the end of feast phase, and 192 mgCOD $gCOD^{-1}$ h^{-1} in the test conducted with biomass sampled at the end of cycle (on average, by considering all batch tests).

A different OUR trend was observed when the biomass was sampled at the end of the SBR cycle and maintained under additional starvation for 2 hours: the endogenous metabolism was characterized by lower values, probably due to the polymer consumption during aeration; after the substrates addition, the OUR strongly increased in the first 20 minutes and it increased more slowly in the time, showing a trend between the storage and growth response. As a result, the specific storage rate was lower than those observed in the tests mentioned above (144 mgCOD $gCOD^{-1}$ h^{-1} on average). In the tests conducted by increasing the starvation time (4 h), the OUR showed a stronger autocatalytic trend; this meant that the growth played a more important role as substrate removal mechanism. The storage response occurred too, but it was characterized by specific rate of 101 mgCOD $gCOD^{-1}$ h^{-1} (on average), the lowest value observed.

In all types of test, the maximum polymer content was always lower than 30% COD COD^{-1}, even if the highest values was obtained without any starvation time (27% COD COD^{-1}); the storage yields were in the range from 0.36 COD COD^{-1} (no starvation) to 0.27 COD COD^{-1} (4 h of starvation). This relatively low values were due to the use of only one spike of substrate at a concentration in batch reactor of 700 mgCOD L^{-1}. However, the results showed that it has no substantial effect to take the biomass from SBR at substrate depletion or at the end of the cycle, but further increasing of starvation time made worse the instantaneous storage response of the biomass.

These results confirmed what observed in a previous study (Majone et al. 1996), in which the effect of the starvation on the storage response was investigated by using biomass acclimated at quite lower organic load.

4B.2.3 PHA productivity

The PHA productivity (gPHA L^{-1} d^{-1}) was evaluated by considering either the second stage only and the combination of both second and third stage. The productivity of the only second stage was calculated by considering the value of PHA concentration at the end of feast phase, the SBR volume only and the flow rate of the influent synthetic wastewater.

The decreasing of the feeding frequency until to 3 times (Run I-b) per day maintaining the same short feeding time (10 minutes) led to an increasing of PHA productivity; by setting the cycle length from 2 to 8 h, the PHA productivity increased almost three times, from 0.18 to 0.53 gPHA L^{-1} d^{-1} (figure 4B.8A). However, the latter value was quite low and a process with only SBR without any accumulation stage it could not be view, also considering a PHA content in the biomass lower than 20% COD COD^{-1} (table 4B.1). The increasing of feeding time until 40 minutes had a negative effect in Run I-a: the PHA productivity was the lowest observed (0.14 gPHA L^{-1} d^{-1}) because of the drastic decrease of the storage response.

The productivity of the II and III stage was calculated by considering the maximum reached PHA concentration at the end of batch accumulation test, whereas a plug flow reactor was used as reference model to calculate the third stage volume by taking into account the contact time needed to reach this maximum PHA concentration, and using a wastewater at 8.5 gCOD L^{-1} as substrate.

Based on these considerations, the observed trend of PHA productivity after accumulation was opposite compared to the II stage only (figure 4B.8A), because it decreased by increasing the overall cycle length (from Run III to Run I-b) at the same feeding time. By operating with a cycle length of 2 h (Run III), the PHA productivity increased by a factor of 10 showing the highest value, 1.7 gPHA L^{-1} d^{-1}. In Run II this increasing factor was 2.7 and in Run I-b, only 2.3. The value obtained in Run III was comparable to that reported in a previous paper (Beccari et al. 2010) under the same OLR but without any nutrient limitation. Although the different storage properties of the biomass selected in Run I-b and Run II, the final PHA productivity was similar: this has to be related to the higher non-Polymer biomass concentration in SBR.

Further decreasing in PHA productivity was observed in Run I-a by increasing the feeding time in SBR: only 0.25 gPHA L^{-1} d^{-1} was obtained after accumulation stage; this value was increased less than 2.0 compared to the SBR value. Thus, Run III was the better condition compared to the other investigated in this study, taking into account that after accumulation, the highest PHA productivity was obtained with a PHA content of ~50% on a COD basis (figure 4B.8B).

4B.3 Conclusions

In the perspective of PHA production from organic wastes, the decrease of the overall SBR cycle length determined an increase in polymer production rates. It appeared clearly that until a cycle length of 6 h (feed frequency 4 times per day), the process was feasible at relatively high OLR. At 8 h of cycle length, the biomass storage response was not appropriate; furthermore, an increasing of feeding time maintaining the same cycle length, led to a decrease of the substrate concentration at which the biomass was exposed; this involved in a drastic drop of the storage capacity, even if the feed/cycle length ratio was the same at an overall cycle length of 2 and 8 h. These concepts were easily explained by considering the importance of feast phase (Dionisi et al. 2006; Johnson et al. 2009; Albuquerque et al. 2010; Beccari et al. 2010; Jiang et al. 2011); with a feast phase percentage around 20%, the process was sustainable; its sustainability was compromised for higher values (Valentino et al. 2012). It is also noteworthy, that during the Run II and III, similar performance was obtained even with similarity of the structure of the microbial consortium. The DGGE profiles showed that *Lampropedia hyalina* genus (recently detected as the dominant microorganisms in PHA producing SBRs, Beccari et al. 2009; Beccari et al. 2010) was stably enriched in both runs where the highest storage response was observed, indicating that it was the main PHA storing microorganism of the community. A phylotype closely related to *Meganema perideroedes* and *Thauera*-related phylotype were detected in Run I-a (which presence has been observed in SBR for PHA production, Dionisi et al. 2005b; Dionisi et al. 2006; Beccari et al. 2009); shortening the feeding time (Run I-b) induced the enrichment of a *Hydrogenophaga* sp. strain and *Thauera* phylotype, the most prominent community member at the end of the Run.

The PHA accumulation performance confirmed the importance to carry out the selection stage in a better way, for the choices of cycle length and feeding time. In fact, the polymer productivity in the three-stage process (which depends on both biomass concentration and polymer production rate, Dionisi et al. 2006; Dionisi et al. 2007), reached a maximum value of 1.71 gPHA L^{-1} d^{-1} at an overall cycle length of 2 h sampling the biomass at the end of feast phase; despite of the higher biomass concentration in the SBR run carried out with the overall cycle length of 8 h.

However, in a perspective of selection and PHA accumulation stages implemented continuously in a larger scale, setting the SBR cycle length at 6 h (and discharging the biomass 4 times per day) could simplify the process management, despite of the lower PHA productivity (1.25 gPHA L^{-1} d^{-1}): in fact, a time of 6 h was more than enough in order to reach a PHA biomass saturation, and no starvation time is necessary from the II and III stage. On the other hand, the SBR cycle length of 2 h assured the best storage performance in terms of PHA productivity (the final PHA content was similar in Run II and III after batch tests); however this storage response could be partially lost by setting a starvation time before to begin the accumulation stage.

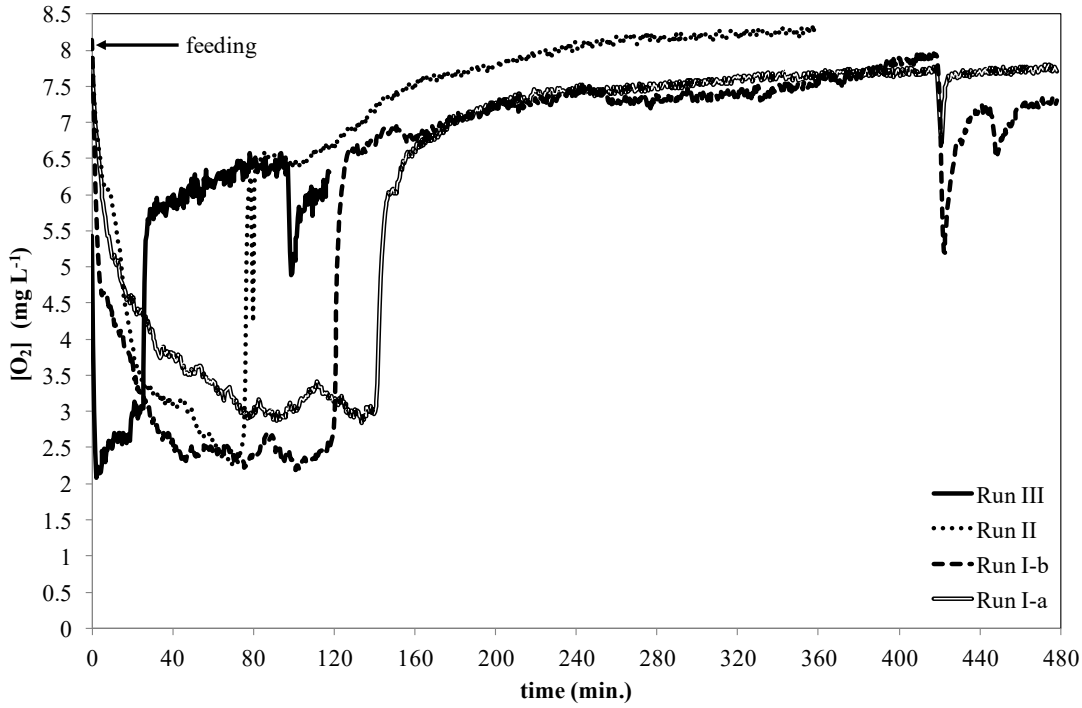
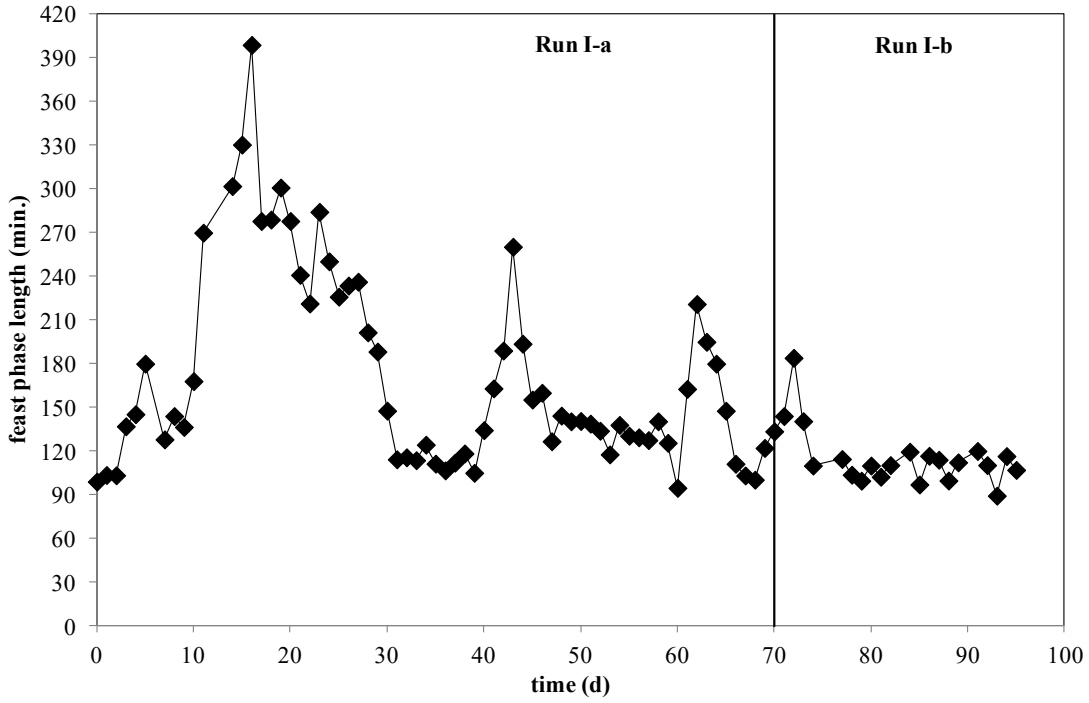
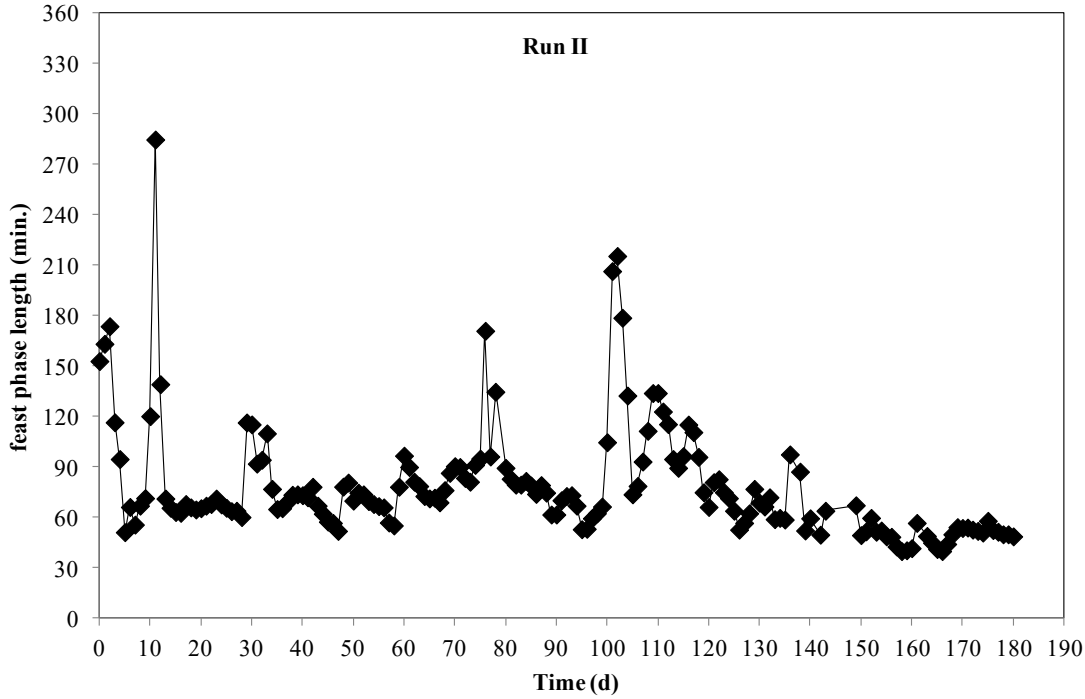


Figure 4B.1. Typical O_2 concentration profiles obtained from automatic data collector during the operating cycles of the different SBR runs.





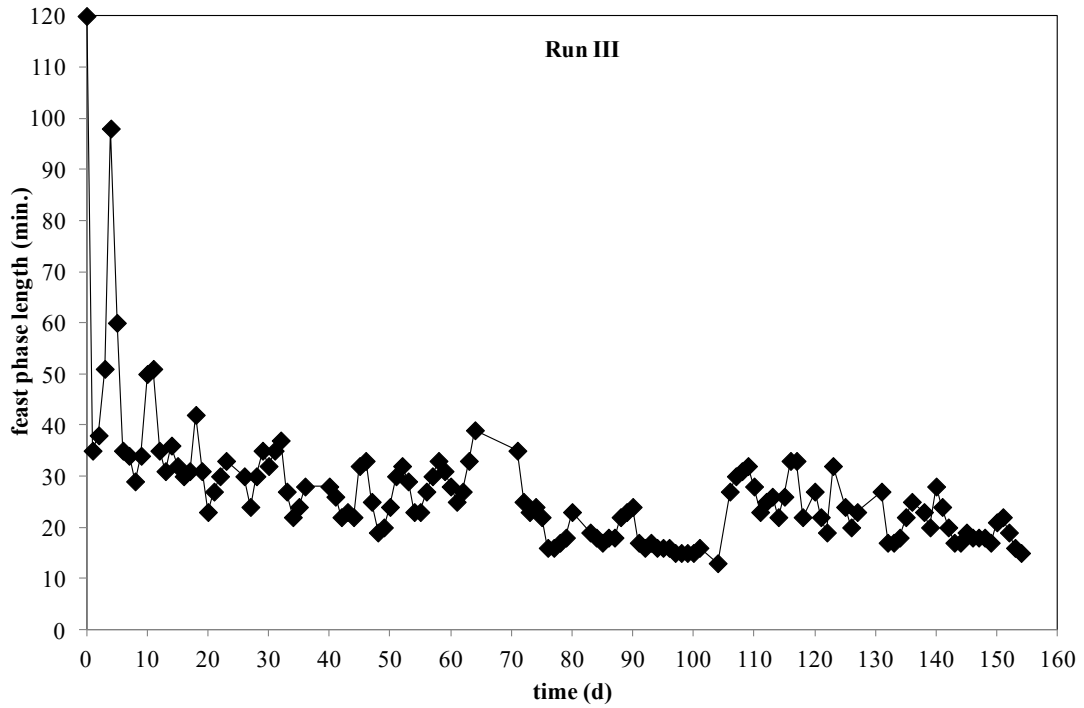


Figure 4B.2. Length of the feast phase during the SBR runs

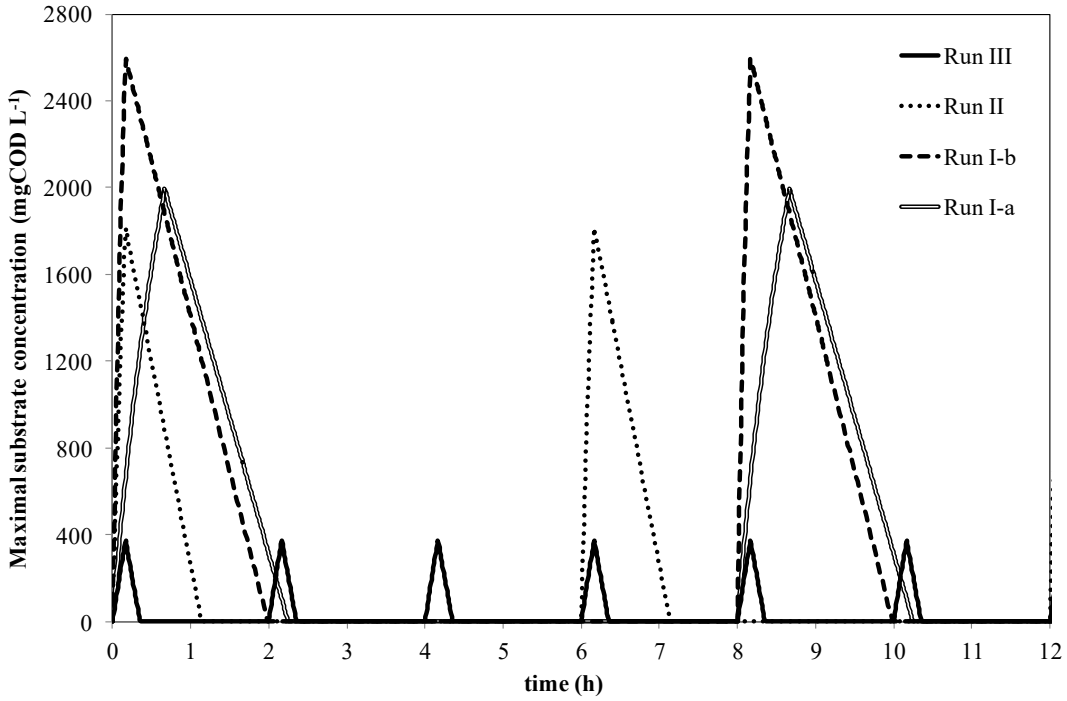


Figure 4B.3. Semi-experimental profiles of the substrate concentration in SBR during the operating cycles of the different SBR runs

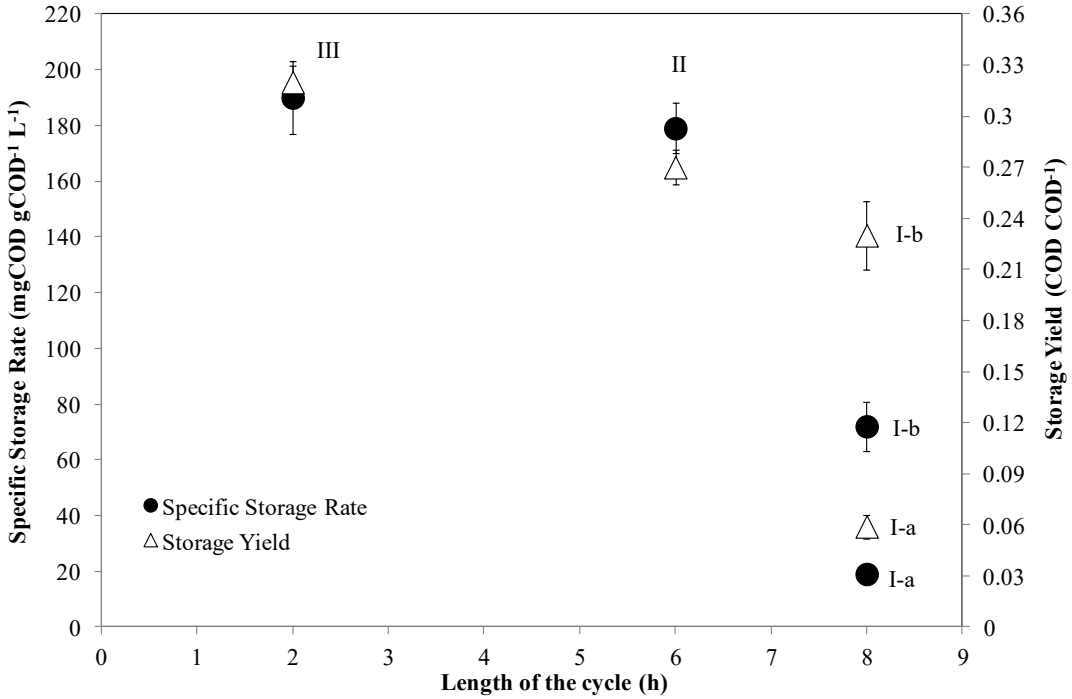


Figure 4B.4. Specific storage rate and storage yield (average values) during SBR runs at different cycle length. Values refer to pseudo-steady state of each run. The number of the corresponding run is indicated in the figure

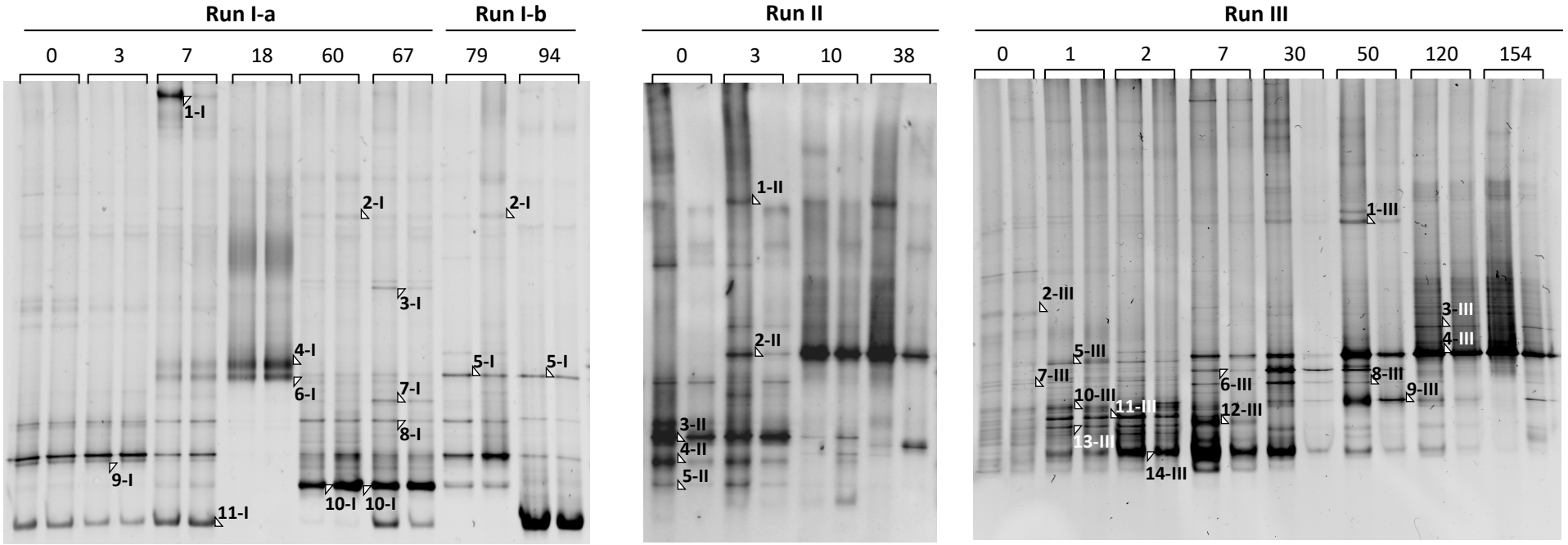


Figure 4B.5. Structure of the bacterial community throughout the long term SBR run

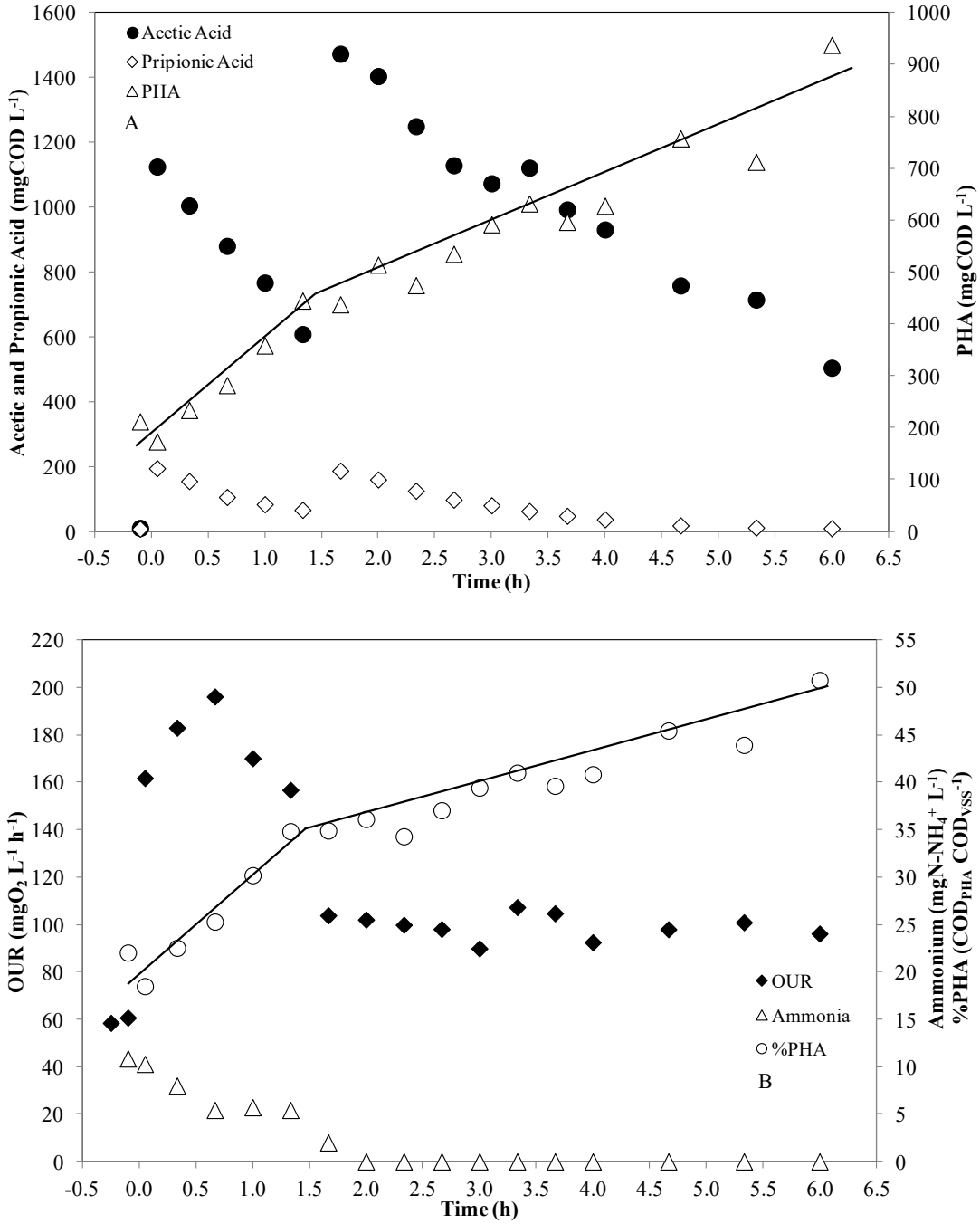


Figure 4B.6. Typical batch tests under partial nitrogen limitation carried out with biomass sampled from SBR Run II: Substrates and PHA (A); OUR, Ammonium and Polymer content in the biomass (B)

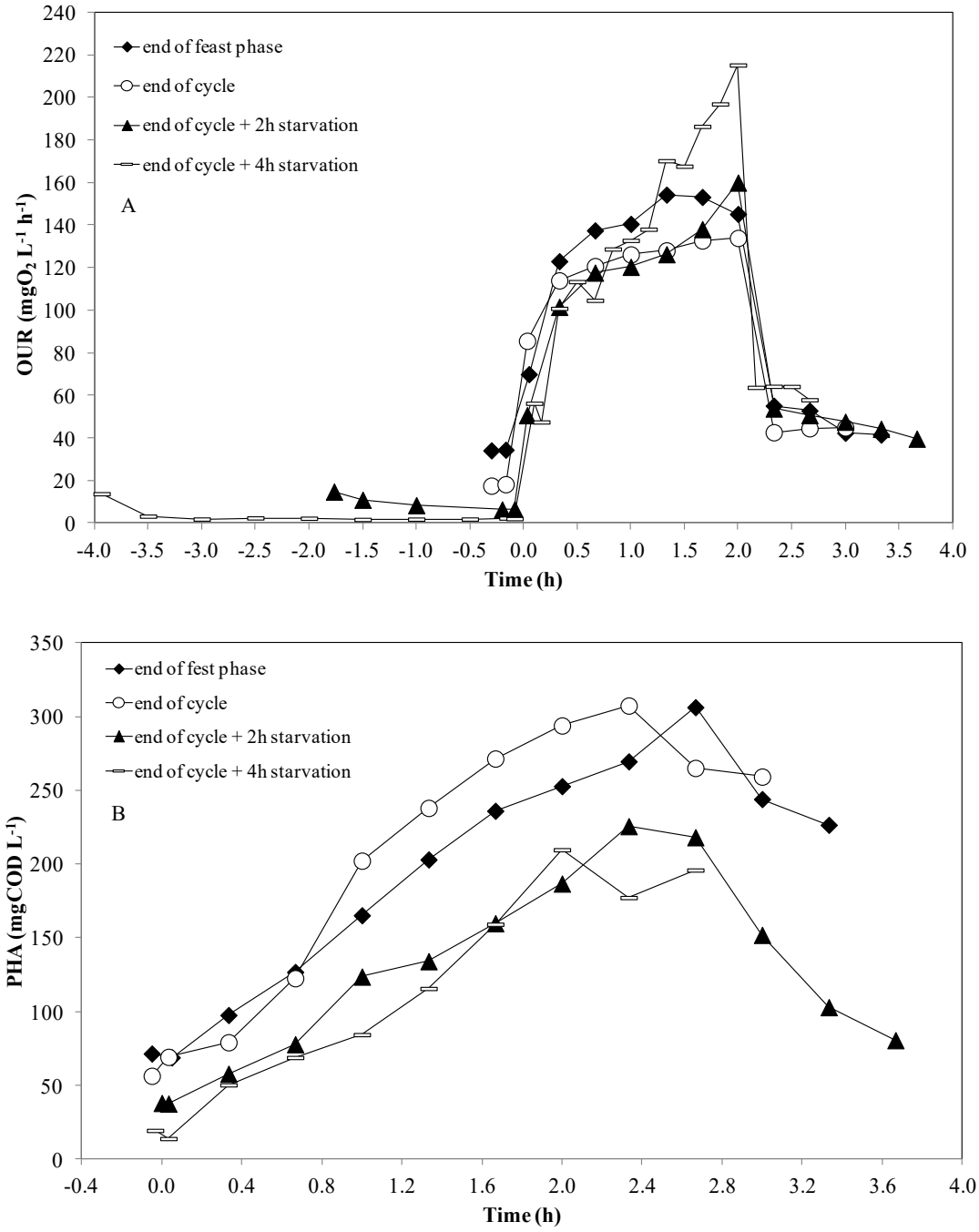


Figure 4B.7. Short-term batch tests with biomass sampled from SBR Run III at different times: OUR (A) and Polymer concentration (B)

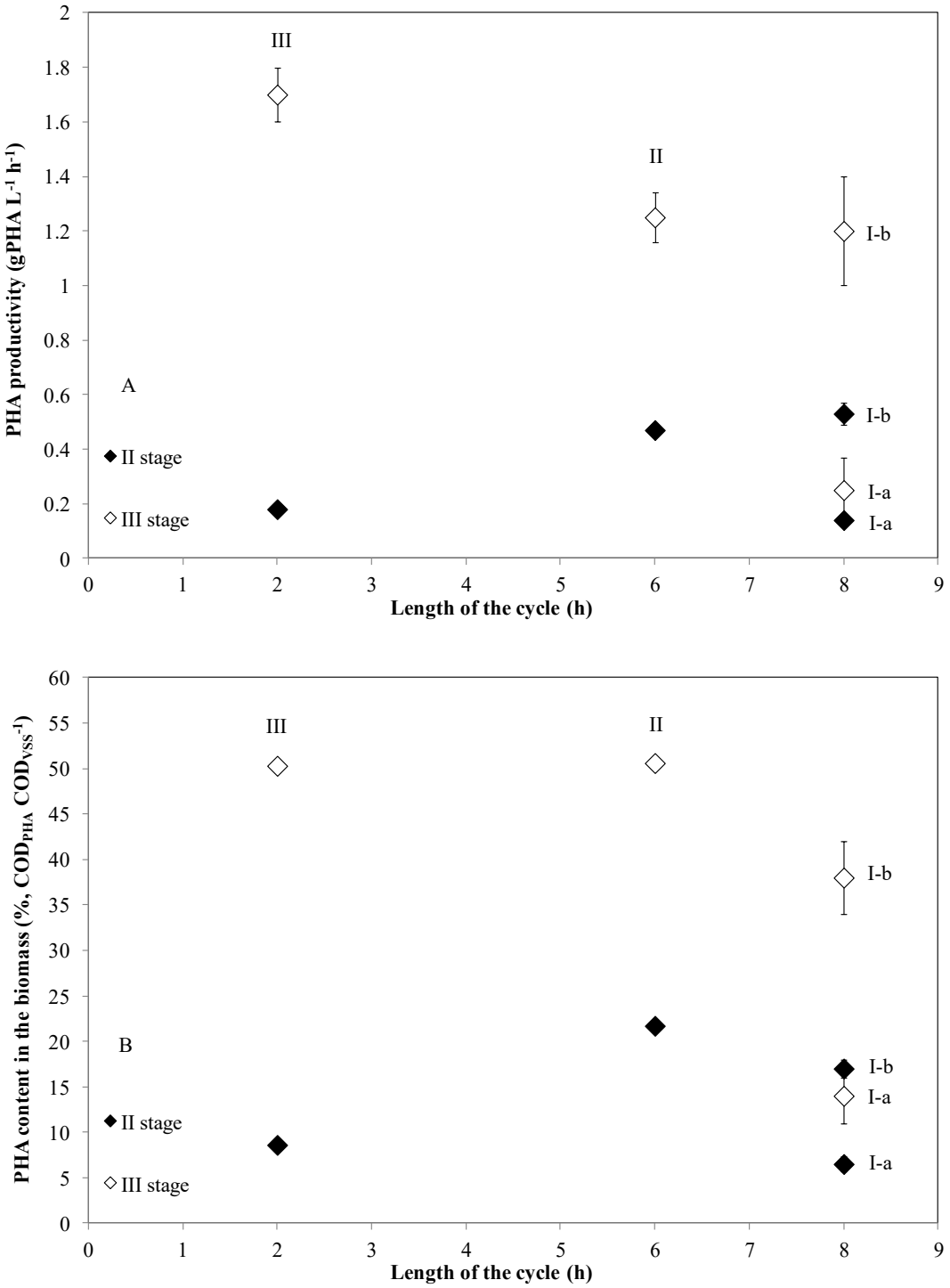


Figure 4B.8. PHA productivity (A) and PHA content in the biomass as percentage in terms of COD (B) of II and III stage in different SBR run

Parameter	Run I-a	Run I-b	Run II	Run III
nonPolymer biomass concentration at substrate depletion (mgCOD L^{-1})	3570 ± 90	4013 ± 212	2810 ± 34	3098 ± 104
PHA concentration at the end of the cycle (mgCOD L^{-1})	92 ± 12	263 ± 26	225 ± 16	124 ± 7
PHA concentration at substrate depletion (mgCOD L^{-1})	238 ± 27	910 ± 70	786 ± 29	374 ± 13
overall cycle length (h)	8	8	6	2
feeding time (min.)	40	10	10	10
time needed for substrate depletion (min.)	157 ± 11	125 ± 7	74 ± 2	23.6 ± 0.6
length of feast phase/length of cycle (%)	33 ± 2	26 ± 1	20.4 ± 0.6	19.7 ± 0.5
specific substrate removal rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	298 ± 25	329 ± 13	659 ± 16	612 ± 26
specific storage rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	19 ± 3	72 ± 9	179 ± 8	190 ± 7
Storage Yield (Y_{STO}^{feast}; COD COD^{-1})	0.05 ± 0.01	0.23 ± 0.02	0.27 ± 0.01	0.32 ± 0.01
Observed Yield (Y_{OBS}^{SBR}; COD COD^{-1})	0.44 ± 0.01	0.55 ± 0.02	0.42 ± 0.01	0.41 ± 0.01
%PHA at the end of feast phase (COD_{PHA} COD_{VSS}^{-1})	6.5 ± 0.8	17 ± 1	21.7 ± 0.7	8.6 ± 0.4
PHA productivity (gPHA L^{-1} d^{-1})	0.14 ± 0.01	0.53 ± 0.04	0.47 ± 0.02	0.18 ± 0.01

Table 4B.1. Average values during pseudo-steady state of measured parameters in SBR runs

Band	Phylogenetic affiliation	Closest relative [accession #]	bp identity	Closest described bacterium [accession #]	bp identity
4-I	Betaproteobacteria	*Alcaligenes faecalis* strain ZJUTBX11 [JF682513]	100 %		
5-I	Betaproteobacteria	*Hydrogenophaga sp.* TR7-01 [AB166886]	99 %		
6-I	Betaproteobacteria	*Alcaligenes faecalis* strain KH-48 [JQ612523]	99 %		
8-I	Firmicutes	*Planococcus sp.* PS30 [GU930769]	99 %		
9-I	Alphaproteobacteria	*Paracoccus denitrificans* strain SDT1S8 [JQ045827]	100 %		
10-I	Alphaproteobacteria	Alpha proteobacterium EU26 [AY428761]	99 %	*Meganema perideroedes* strain Gr2 [AY170117]	99 %
11-I	Betaproteobacteria	Uncultured *Betaproteobacteria bacterium* clone QEDP3BC01 [CU924572]	98 %	*Thauera butanivorans* strain NBRC 103042 [AB681922]	97 %

Table 4B.2-A. Taxonomic characterization of the major bands in the DGGE profiles (Run I-a-b)

Band	Phylogenetic affiliation	Closest relative [accession #]	bp identity	Closest described bacterium [accession #]	bp identity
2-II	Betaproteobacteria	*Lampropedia hyalina* DSM 15336 [AY291119]	100 %		
4-II	Betaproteobacteria	*Hydrogenophaga flava* strain ENV735 [GQ404493]	99 %		
5-II	Alphaproteobacteria	*Defluvibacter sp.* QDZ-C [HQ890470]	100 %	*Defluvibacter lusatiensis* strain LQ-H1 [EU870446]	100 %
6-II	Alphaproteobacteria	*Uncultured Xanthobacter sp.* clone EK_CK546 [JN038223]	99 %	*Xanthobacter autotrophicus* strain ce41 [JN082248]	98 %

Table 4B.2-B. Taxonomic characterization of the major bands in the DGGE profiles in Run II

Band	Phylogenetic affiliation	Closest relative [accession #]	bp identity	Closest described bacterium [accession #]	bp identity
4-III	Betaproteobacteria	*Lampropedia hyalina* DSM 15336 [AY291119]	100 %		
10-III	Betaproteobacteria	*Acidovorax defluvii* (T) BSB411 [Y18616]	99 %		
11-III	Betaproteobacteria	uncultured bacterium 120ds10 [AY212571]	100 %	*Dechloromonas* sp. EMB 50 [DQ413149]	99 %
13-III	Betaproteobacteria	uncultured bacterium Z-62 [FJ901124]	98 %	*Dechloromonas* sp. EMB 269 [DQ413167]	97 %
14-III	Betaproteobacteria	*Thauera selenatis* (T) ATCC 55363T [Y17591]	100 %		

Table 4B.2-C. Taxonomic characterization of the major bands in the DGGE profiles in Run III

Parameter	Run I-a	Run I-b	Run II	Run III
specific substrate removal rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	221 ± 43	252 ± 67	624 ± 42	787 ± 162
specific storage rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	34 ± 11	115 ± 10	216 ± 17	196 ± 13
Storage Yield (Y_{STO}^{feast}; COD COD^{-1})	0.06 ± 0.02	0.33 ± 0.02	0.45 ± 0.01	0.53 ± 0.01
%PHA at the end of the test (COD_{PHA} COD_{VSS}^{-1})	14 ± 3	38 ± 4	50.6 ± 0.3	50.3 ± 0.7
PHA productivity (gPHA L^{-1} d^{-1})	0.25 ± 0.12	1.2 ± 0.2	1.25 ± 0.09	1.7 ± 0.1

Table 4B.3. Main parameters (average values with relative standard deviation) collected from batch tests carried out with acclimated biomass sampled from different SBR Run

CHAPTER 5. DEVELOPMENT OF A LAB-SCALE MULTI-STEPS CONTINUOUS SYSTEM : II AND III STAGES OF PHA PRODUCTION PROCESS PLUS IV STAGE FOR QUENCHING AND PHA EXTRACTION

A lab-scale continuous system was implemented based on the results obtained from previous experiments (chapter 4B). The experimental set-up consisted of:

- II stage in SBR for biomass selection and production;
- III stage for PHA accumulation by using the biomass selected and produced in the II stage;
- IV stage for quenching of biological reaction and PHA extraction.

These stages were continuously performed (figure 5.1).

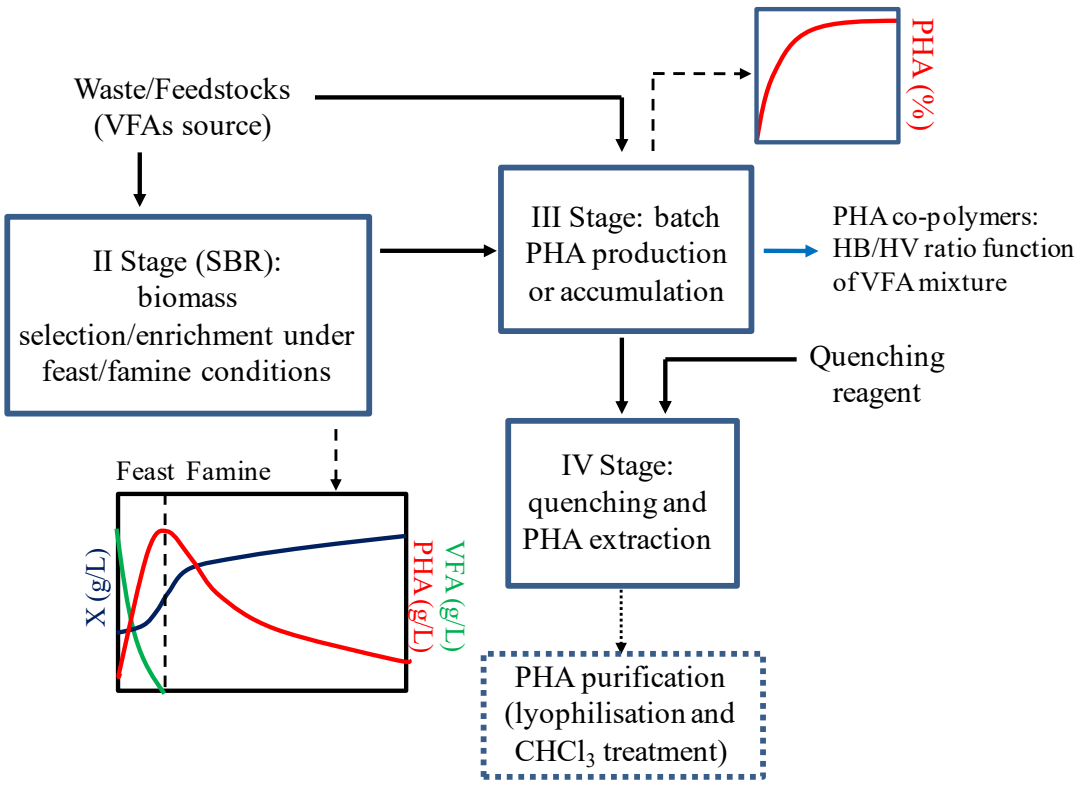


Figure 5.1. Scheme of the lab-scale PHA production process developed in a continuous way, for the II, III and IV stages. The final PHA purification phase was made separately.

More in detail, the selected/enriched biomass from the II stage (SBR) was withdrawn in a batch reactor without any dilution, for the III stage (PHA accumulation). The latter stage was conducted at higher load than II stage, under nitrogen limitation condition. In addition, the system of quenching/extraction was implemented in a continuous way as well, in order to stop all biological reaction and collect the whole polymer produced previously.

Various recovery technologies have been proposed and studied in small scales in the laboratory as well as in industrial scales, such as solvent extraction, chemical digestion, enzymatic treatment and mechanical disruption, supercritical fluid disruption, flotation techniques (Kunasundari and Sudesh, 2011). The chemical digestion methods is based on the solubilisation of non-PHA cellular mass (NPCM) and mainly utilizes sodium hypochlorite as strong oxidizing reagent (Yu and Chen, 2006) or surfactants such as sodium dodecyl sulphate (SDS) even in combination (Ramsay et al. 1990), in order to overcame the problems linked to the purity and to the molecular weight degradation using either surfactant or sodium hypochlorite alone (Yu, 2009). Hahn and co-researchers established a separation process that took advantage of both differential digestion and solvent extraction (Hahn et al. 1993; Hahn et al. 1994). The hydrophobicity of P(3HB) and hydrophilicity of lyophilized cells accounted for the development of dispersions of a sodium hypochlorite solution and chloroform. A study by Valappil et al. (2007) described that the high molecular weight of P(3HB) could be retained by using this method. The main limitation is the use of large quantity of solvent that would raise the recovery cost.

The strategy chosen in this process for the PHA extraction was the chemical digestion, firstly carried-out with sodium hypochlorite (active Cl_2 5%) as oxidizing reagent; NaOH 1 M as basifying agent was also used for a comparison of a different extraction strategy.

The effluent from the IV extraction step was then collected and centrifuged in order to collect residual solids (containing PHA) and to characterized them. On some of collected samples, a further step of biomass lyophilisation was performed before the final $CHCl_3$ treatment, in order to complete the product purification and to obtain a polymeric PHA film.

5.1 Materials and methods

5.1.1 II stage of the process (SBR)

The aerobic enrichment of the PHA-producing biomass was performed in a 1 L working volume SBR that was inoculated with the activated sludge from the "Roma Nord" full-scale WWTP. Before inoculum, the sludge was left to settle in order to remove the supernatant, and resuspended in a synthetic mineral medium (whose the composition is given in paragraph 4A.1.1). Then, the sludge was aerated for at least one night before the inoculum.

The SBR was fed with a mixture of acetic and propionic acid (85% and 15% on a COD basis, respectively, at an overall concentration of 8.5 gCOD L^{-1}). The SBR was operated at 4 cycles per day (cycle length 6 h). By considering the mean value of the biomass (table 1, chapter 4B), the theoretical S/X ratio in each cycle was ~0.6 on a COD basis. The substrate feeding and the mixed liquor discharge were made for the first 10 minutes and for last 1 minute of each cycle. The latter was fully transferred to the next step. Overall, the SBR was operated at OLR of 8.5 gCOD L^{-1} d^{-1} and HRT of 1 day without settling phase (SRT equal to HRT).

The SBR run was characterized by a long period (~150 days) in order to better investigate the acclimated biomass performance.

The system was set at the same operating conditions already described in 4A.1.1, as well for the mineral medium composition and sampling activity.

During each SBR run, it was considered that a pseudo-steady state was obtained when feast phase length remained approximately constant (within 5% deviation from average values) for at least 5 consecutive days.

5.1.2 III stage of the process (PHA accumulation)

The III stage of the process was conducted in a 0.5 L of reactor at the same temperature of SBR (25°C) by using a water bath. The reactor was mixed by magnetic stirrer and aerated by means of membrane compressors at high flow rate (in order to maintain aerobic condition). The dissolved oxygen (DO) concentration was controlled by using DO probe (CellOx 325 WTW). Aeration and stirring were high enough to maintain DO above 2 mg L^{-1}.

The organic feed was prepared in a mineral medium, having the same composition of that used for SBR (paragraph 4A.1.1) but without any nitrogen source (thus limiting cellular growth); thus, the only nitrogen content was the residual amount in the effluent from SBR. The VFAs concentration in the feed was 37.12 and 6.54 gCOD L^{-1} for the acetic and propionic acid respectively. This concentration (much higher than in the SBR feed concentration) was necessary in order to achieve a S/X ratio equal to ~2.0 on a COD basis, ~3.4 times higher than in the SBR. The nitrogen limitation imposed to this stage, favored the storage phenomena as the most relevant mechanism of substrate uptake. The pH of this feeding solution was adjusted at 6.0 by adding NaOH 3M.

At any SBR cycle, the mixed liquor was withdrawn from SBR, close to the substrate depletion. Hence, 0.25 L of sludge was loaded in the III stage in 1 min.; immediately after, 0.05 L of the feeding solution was added in 30 min. The overall III stage length was 6 h (equal to SBR cycle length). At the end of the III accumulation stage, the PHA rich sludge was automatically transferred to the following step. Several times per week, PHA, TSS and VSS samples were taken immediately before. The sludge was withdrawn towards next step.

During the kinetics characterization of PHA storage and VFA uptake, the liquid phase was sampled for VFA and ammonia measurements and the sludge was sampled for PHA measurements at regular intervals. The OUR measurements were made before feeding, at the end of feeding (30 min.) and at regular intervals until the end (6 h).

5.1.3 Quenching and PHA extraction

At the end of each accumulation stage, the biomass with high PHA content was discharged in a 5 L quenching reactor, in which a solution inhibiting the biomass activity was added, in order to prevent the PHA consumption. After quenching, the biomass was maintained in the reactor for obtaining the destroy of cell walls and release of PHA. Then, the sludge was centrifuged (8600 rpm for 30 min) and stored at -20°C until the lyophilisation and purification.

NaClO 5% active Cl_2 or NaOH 1M as alternate quenching solutions were used, both of them were added in order to obtain a 5:1 v/v ratio of sludge/solution. The quenching solution addition was made simultaneously to the PHA rich sludge withdrawal from the III stage. The treated biomass was daily centrifuged, collected in plastic tube and preserved at -20°C until the following lyophilisation and purification steps, carried out manually respect to the whole process. For this reason, all the PHA rich sludge was maintained in contact with the quenching solution for the whole week-end, until the centrifugation made in the first working day of the week. The PHA and TSS sampling was done after 3 and 24 h passed from quenching solution addition. A filter of low porosity (0.2µm) was used for TSS measurements, in order to avoid loss of solid (treated biomass) in the filtered volume. The filters were made of organic matter and they were not adapted for VSS measurements at high temperature (550°C).

The biomass treated with quenching solution, was firstly defrozen and then washed with distilled water, centrifuged (6000 rpm for 30 min) and lyophilized (T -40°C, pressure 5 mmHg, 40 h). The lyophilized biomass was suspended in $CHCl_3$ (~38 mL of $CHCl_3$ per gram of lyophilized biomass) at 37°C for 3 days. Then, the sample was filtered on glass fiber filter (discharging the non-soluble matter) by using vacuum pump (400 mbar) and left under fume hood for $CHCl_3$ evaporation obtaining a polymeric film. The lyophilized sample (~0.5 g) and the film sample were analyzed on GC (4A.1.3).

5.1.4 Analytical methods

See paragraph 4A.1.3

5.1.5 Calculations

In the SBR, all the parameters were calculated as described in the paragraph 4A.1.4; the III accumulation stage was equivalent to long-term batch tests under nitrogen limitation in terms of calculations (4A.1.4).

5.2 Results

5.2.1 II Stage of the process (SBR performance)

The results showing the SBR performance are already explained in paragraph 4B.2.1. The storage response of the selected biomass is quantified by the parameter reported in the Table 4B.1 in the column Run II (chapter 4B).

5.2.2 III Stage of the process (accumulation performance)

The III stage in continuous mode was started after 57 days from initial inoculum of SBR. Figure 5.2 shows the PHA concentration trends in the SBR (at substrate depletion, that also corresponded to the influent to the III stage), and at the end of III stage (after 6 h of accumulation). A substantial increase of PHA concentration was reached after accumulation, except for two defined windows only: a) in the first days in which the III stage was implemented, probably because an oxygen limiting condition due to not sufficient aeration of the biomass (that was not diluted from SBR); b) from the 104th to 115th day, because of a quite low selective pressure imposed to the biomass in the SBR (high feast phase length, figure 4B.1, Run II, chapter 4B).

On average, the PHA concentration increased of 1.3 gPHA L^{-1}; also the PHA content in the biomass increased from 18% (II stage) to 49% (III stage) gPHA gVSS^{-1} on average. (the average value was calculated from the 90th day when the TSS/VSS sampling was set, figure 5.3).

The increase of the VSS and TSS concentration at the end of accumulation were 1.2 and 1.3 g L^{-1} higher than the relative values in SBR, respectively, and it was attributed to the PHA production. By taking into account the flow rate of third stage (1.2 L d^{-1}), a polymer production of 2.1 ± 0.1 gPHA d-1 at the of the accumulation was reached.

In table 5.1, the results of the III stage kinetics characterization are reported. The tests were conducted around the 170th day of process operation; at that time, the short feast phase monitored in SBR (~15%) showed a good selective pressure on the biomass (Beccari et al. 1998; Dionisi et al. 2004; Serafim et al. 2004; Dionisi et al. 2005b; Dionisi et al. 2006; Dionisi et al. 2007; Johnson et al. 2009; Albuquerque et al. 2010; Beccari et al. 2010; Bengtsson et al. 2010; Jiang et al. 2011).

The kinetic tests showed a good reproducibility of the results and also a good storage performance of the biomass: the initial specific storage rate was comparable to the initial specific substrate uptake rates; as a consequence, the initial storage yield was 0.84 COD COD^{-1} at least at the beginning. At the end of the tests, the storage yield was 0.60 ± 0.04 COD COD^{-1} and the final polymer content in the biomass was 61 ± 2 % gPHA $gVSS^{-1}$.

Specifically, figure 5.4 (A-B-C) shows the main parameters monitored during one kinetic characterization of the accumulation stage. Because of the high concentration of both substrate addition and biomass (not diluted from SBR), the OUR increased to values much higher than endogenous: 230 mg O_2 L^{-1} h^{-1} at the end of feeding phase (0.5 h) and ~500 mg O_2 L^{-1} h^{-1} at 1 h. Because of the total nitrogen uptake after 1 h (the initial $N\text{-}NH_4^+$ was ~30 mg L^{-1}), the OUR decreased until to ~400 mg O_2 L^{-1} h^{-1} remaining then almost constant until the end of the accumulation step. The OUR trend after nitrogen uptake was not autocatalytic, indicating the storage activity as the main mechanism of substrate uptake. In order to reach the PHA saturation, 4 h seemed to be enough because of the high value of PHA content (~64% gPHA $gVSS^{-1}$) and no further increase in the 2 h remaining (figure 5.4C). The same trend was observed for the PHA concentration: most of the production was in the first 4 h when both acids were consumed at the same time (figure 5.4B). In the last 2 h, the PHA concentration did not increase; however, the VFAs continued to be consumed, but only acetic acid because of the lack of propionic acid (almost totally consumed in the first 4 h). The HV percentage, which value only slightly increased until 4 h (when propionic acid was still not totally removed) from initial 15% to ~20% gHV $gPHA^{-1}$, drastically decrease to 15.8% in the last 2 h of accumulation according to the lack of propionic acid (figure 5.4C).

In figure 5.5 the main mechanisms of substrate uptake are reported in terms of single contribution to the overall COD consumption. Each contribution (storage, oxidation and growth) was measured separately and independently: the storage was calculated from polymer analysis, the oxidation from OUR measurement, the growth from nitrogen consumption. The sum of each contribution was perfectly comparable to the total COD consumption (derived from VFAs uptake).

This meant that all important mechanisms contributing to the substrate uptake had been taken into account. As expected, the storage contribution was the main mechanisms (62% in terms of COD removed) followed by oxidation (32%) and growth (5.8%).

5.2.3 Quenching performance and PHA extraction

At first sight, the NaClO treatment was a better way to recovery the polymer from the accumulation stage. As reported in table 5.2, the concentration of PHA decreased of 11% after 3 h, and almost 20% after 24 h of a NaClO treatment. On the other hand, this decrease was 22% and 30% after 3 and 24 h respectively by using NaOH. However, part of this concentration decrease is also due to dilution for the addition of the extracting agents. In terms of mass balance, it is also necessary to consider the flow rate of the accumulation stage (1.2 L d^{-1}) and quenching/extraction stage (1.4 L d^{-1}) in order to have a more precise the recovery performance as mass flow: indeed, the loss of PHA after 24 h of NaClO treatment was only 6% (2.06 gPHA d-1 at the end of III stage, 1.93 gPHA d^{-1} after 24 h extraction). Already after 3 h, the NaOH treatment occurred in a PHA loss of ~10%, and 18% after 24 h. The NaClO treatment occurred not only in a almost total PHA recovery but also in a substantial decrease of TSS: 52% and 59% of solids were lost after 3 and 24 h respectively. This involved in an increase of PHA content, that was equal to 49% gPHA $gVSS^{-1}$ at the end of accumulation stage whereas it was 90% and 98% gPHA $gVSS^{-1}$ after 3 and 24 h of NaClO treatment.

By using NaOH as quenching/extraction solution, the TSS decrease was quite less than in the NaClO treatment, having only 37% of solids loss after 24 h of NaOH treatment. By considering also a PHA recovery lower than NaClO treatment (87% and 80% after 3 and 24 h respectively), the PHA content in the biomass increased, if compared to the end of III stage, even though it anyway was lower than 60% gPHA $gVSS^{-1}$.

The higher PHA recovery and higher PHA content (98% gPHA $gVSS^{-1}$) suggested the NaClO treatment as better quenching/extraction strategy in the perspective of downstream processing.

After quenching/extraction, the recovered biomass was subjected to two further purification phases (in a discontinuous mode on selected samples). A PHA powder was obtained from lyophilisation whereas a PHA film was obtained after dissolution and precipitation from $CHCl_3$. The lyophilized powder and polymeric films were analyzed in order to quantify the PHA composition (HB/HV content) and purity (%). Eleven lyophilisation and chloroform purification cycles were carried out (collecting treated biomass from the 59th to 122th day of process operation): the PHA purity (gPHA $gTSS^{-1}$) in the solid lyophilized samples and in the polymeric films is reported in figure 5.6A; the %HV (gHV $gPHA^{-1}$) in the copolymer P(3HB-3HV) obtained in the lyophilized and films samples is reported in figure 5.6B. On average, the % of PHA was higher in the film samples (more purified product, 92.7%) than in the lyophilized powder (89.6%). This also meant that these last steps did not increase the purity of the final product; 98% gPHA $gTSS^{-1}$ after 24 h of NaClO treatment for quenching/extraction, was the highest purity value. In terms of composition, the HV percentage decreased from 9.40% to 7.74% gHV $gPHA^{-1}$, from the lyophilized samples and to the films.

5.3 Conclusions

The storage performance of the SBR was explained in the paragraph 4B.2.1 (Run II): the imposed feast and famine regime occurred in a good storage response; the feast phase percentage was 20.4 ± 0.6 %, the specific storage rate was 179 ± 8 mgCOD $gCODnonPolym^{-1}$ h^{-1}, the storage yield was 0.27 ± 0.01 COD COD^{-1}. With overall SBR cycle length of 6 h, the III stage was implemented in continuous mode with the same time that was enough to reach polymer saturation of the biomass, directly loaded from the SBR without any dilution. The kinetic characterization of the III stage showed a very high storage ability of the biomass: the specific storage rate and the storage yield were 626 ± 16 mgCOD gCODnonPolym^{-1} h^{-1} and 0.60 ± 0.04 COD COD^{-1} respectively, with a final PHA content in the biomass of 61 ± 2 gPHA $gVSS^{-1}$. However, it should be taken into account that a different and more concentrated than SBR synthetic feed was used (43.66 $mgCOD_{TOT}$ L^{-1}), and this could be not feasible in the perspective of a large-scale process.

For extraction and purification steps, the NaClO (5% active Cl_2) treatment was more efficient than NaOH treatment, because of the higher TSS lowering (59%) and PHA recovery (almost 100%), that occurred in a further increasing of the PHA content (98% gPHA $gVSS^{-1}$). In terms of composition, the final HV percentage decreased from 9.40% to 7.74% gHV $gPHA^{-1}$ in the lyophilized samples and in the films respectively.

Figure 5.7 shows the complete scheme of the proposed process: the "in" and "out" flow rate of each stages continuously rurring were taken into account for the calculation of daily PHA production (from 0.42 to 1.93 g d^{-1} at the end of quenching/extraction stage, with 98% of content).

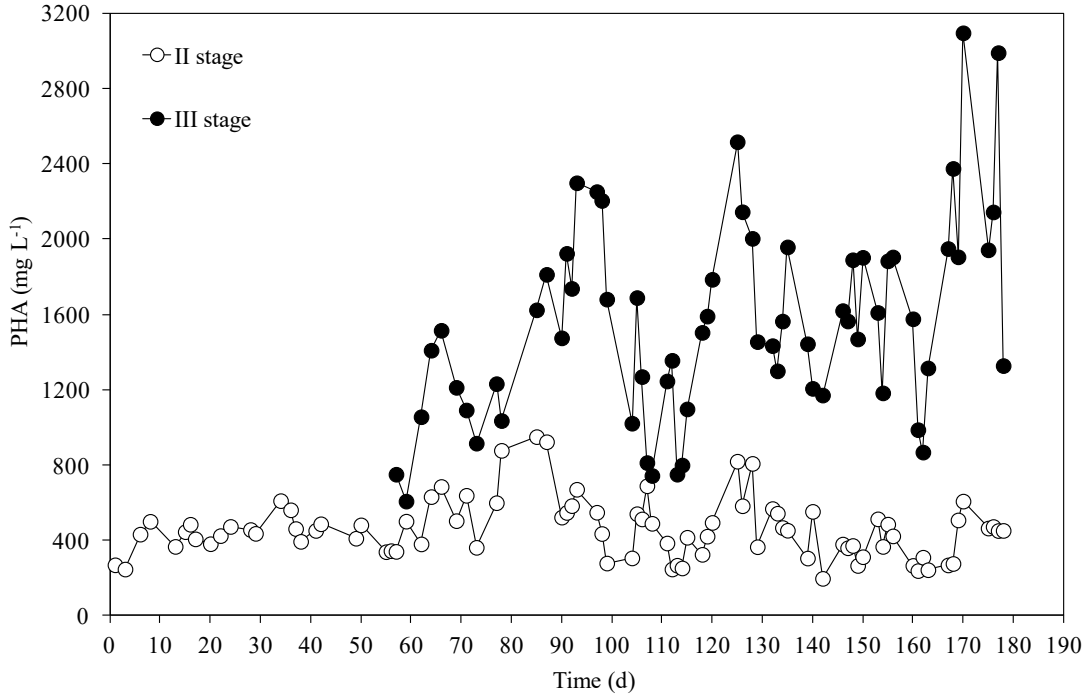


Figure 5.2. PHA concentration in II stage (at the end of feast phase) and at the end of III stage

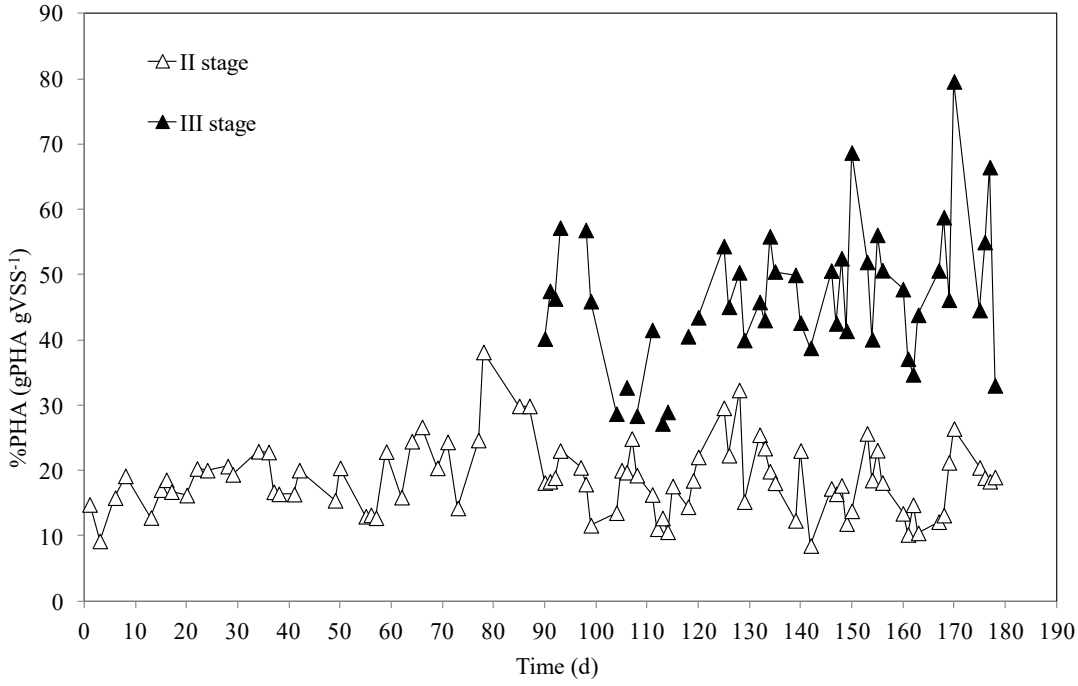
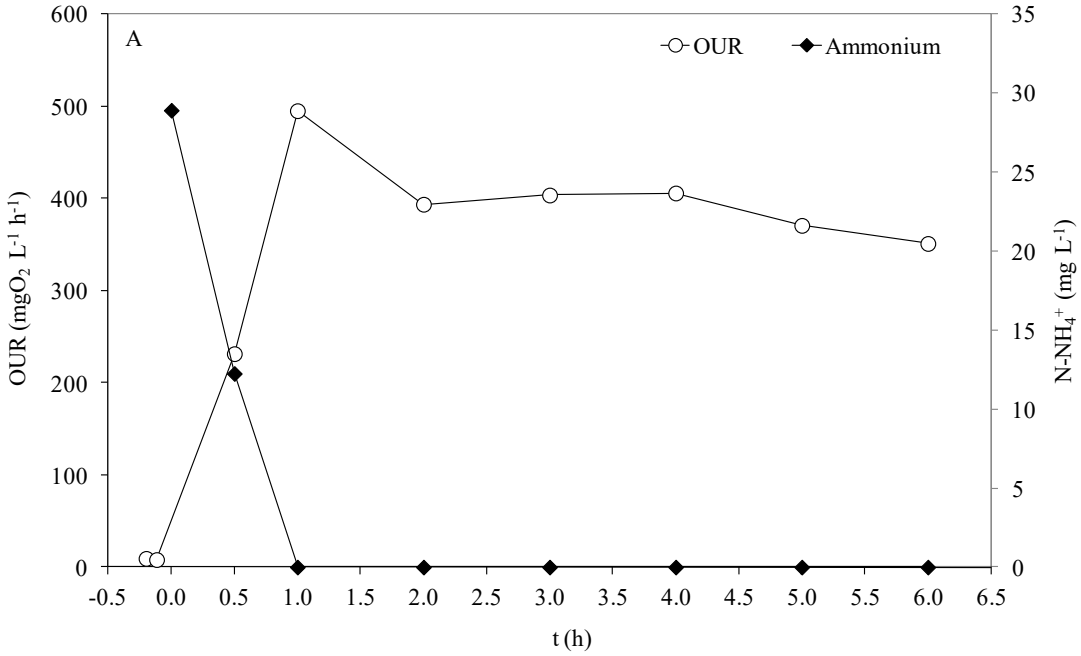
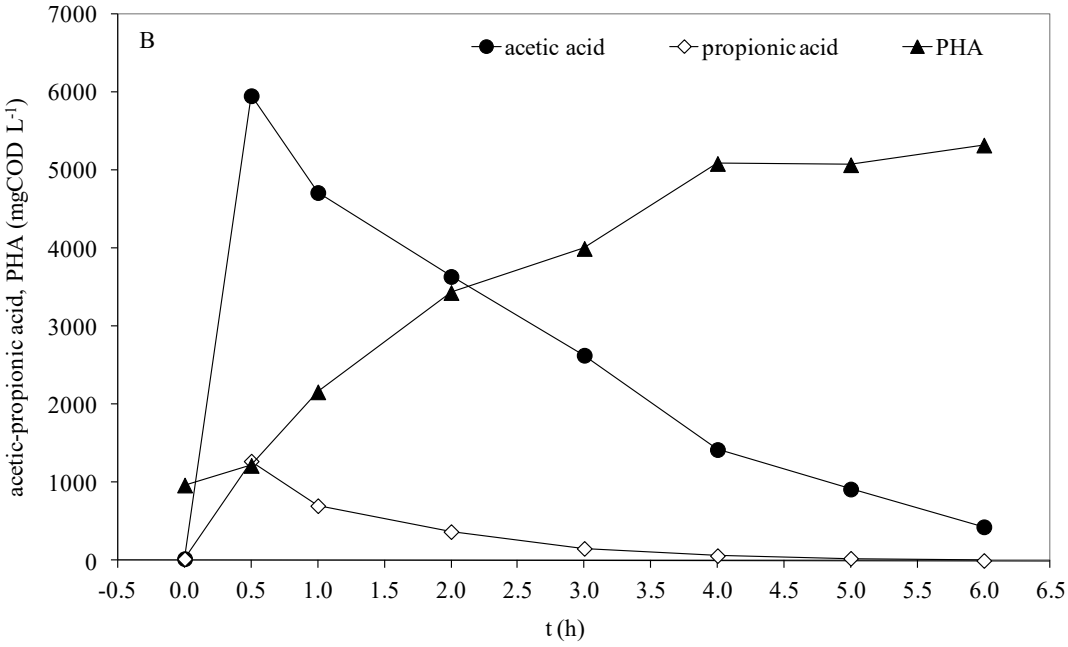


Figure 5.3. PHA content in the biomass in II stage (at the end of feast phase) and at the end of III stage


A
OUR
Ammonium
OUR (mgO2 L-1 h-1)
N-NH4+ (mg L-1)
t (h)


B
acetic acid
propionic acid
PHA
acetic-propionic acid, PHA (mgCOD L-1)
t (h)

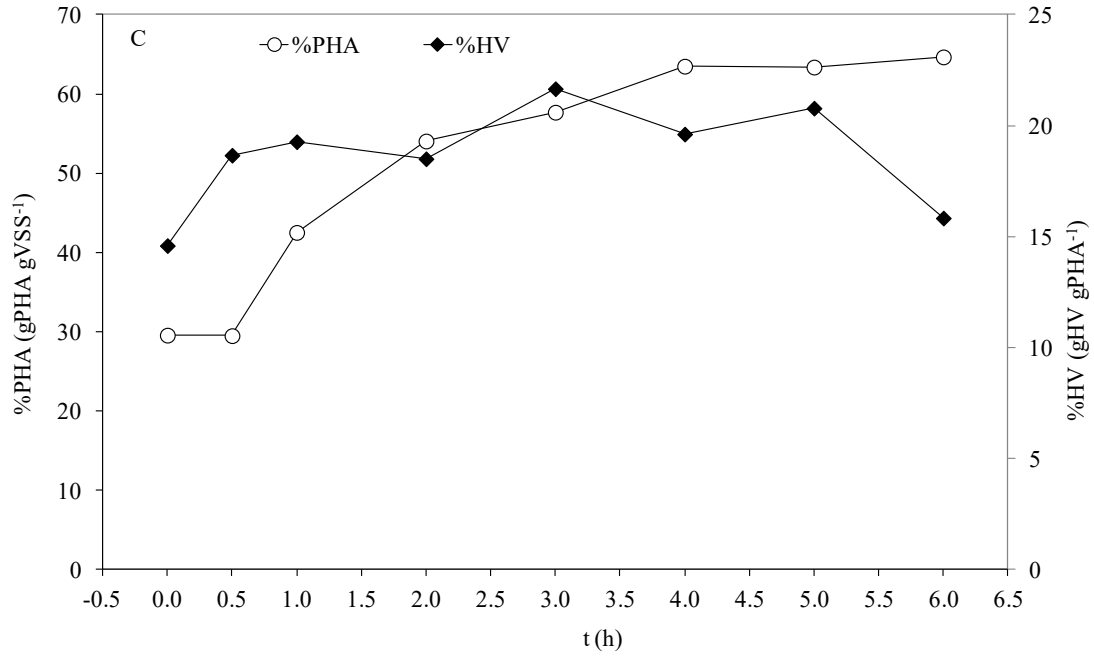


Figure 5.4. Main parameters monitored in a kinetic characterization of the III stage. OUR, Ammonium (A); VFAs and PHA (B); PHA content in the biomass and %HV in the produced PHA (C)

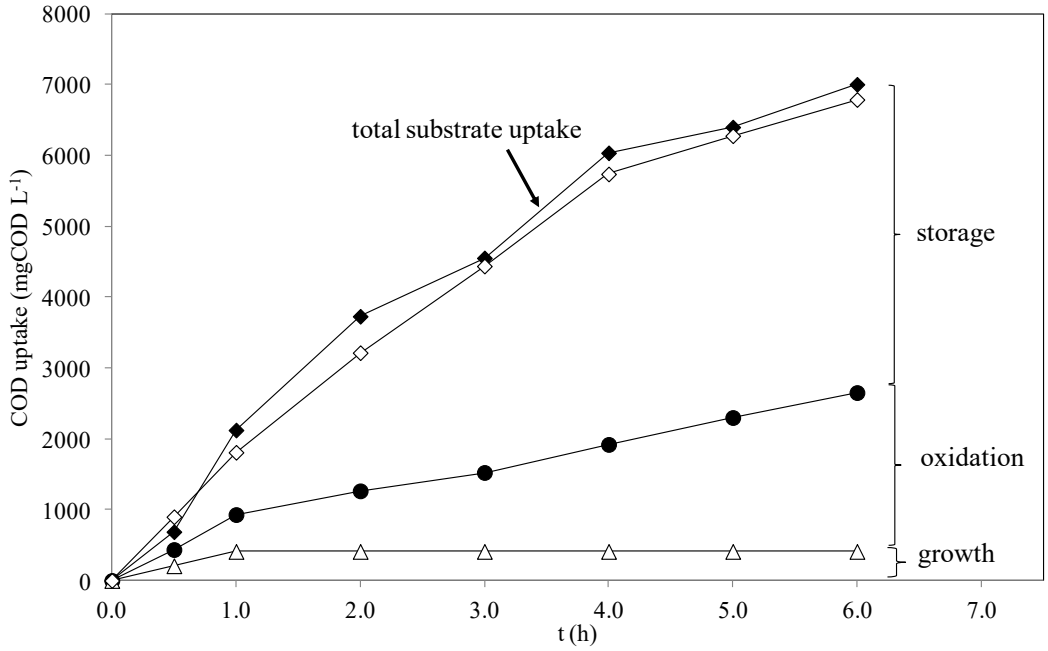


Figure 5.5. Mechanisms of substrate uptake, contribution to the COD removed

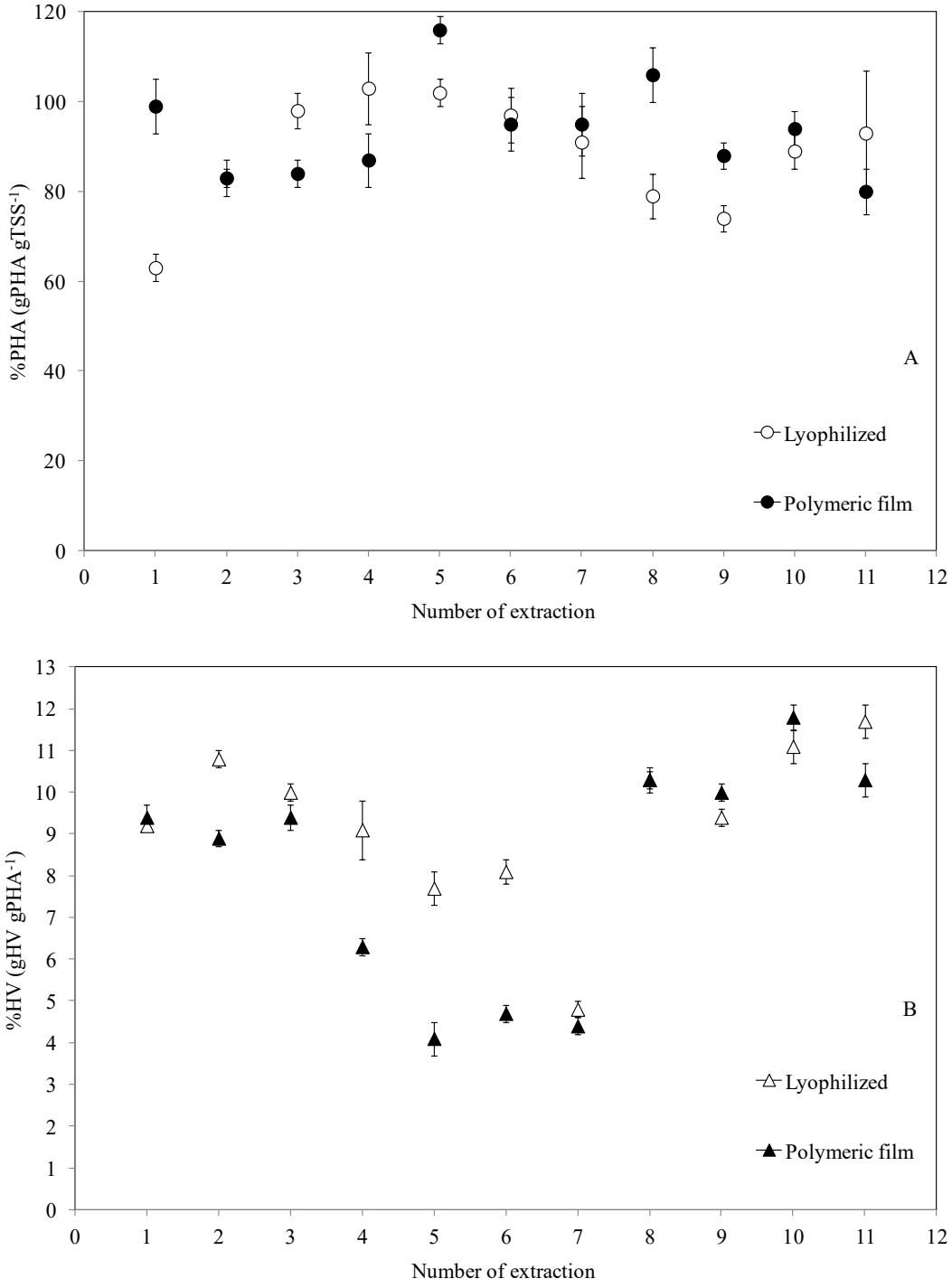


Figure 5.6. %PHA (A) and %HV in the copolymer (B) obtained in the lyophilized and polymeric films samples

Parameter kinetic characterization of the III accumulation stage	**Test I**	**Test II**	**Test III**	**mean value**
Day of the SBR run (from the inoculum)	168	170	171	-
feast phase length (biomass sampling cycle, min.)	50	56	54	53 ± 2
feast/famine ratio (biomass sampling cycle, %)	13.9	15.6	15	14.8 ± 0.5
initial specific substrate removal rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	764	803	648	738 ± 47
initial specific storage rate (mgCOD gCODnonPolym.$^{-1}$ h^{-1})	651	615	612	626 ± 16
Final polymer content (%, gPHA gVSS^{-1})	58	64.7	61.5	61 ± 2
Final HV content (%, gHV gPHA^{-1})	12.3	15.8	16.9	15 ± 1
Final Storage Yield ($Y_{STO;\ COD\ COD}^{-1}$)	0.52	0.64	0.63	0.60 ± 0.04
Final PHA concentration (mg L^{-1})	2168	2545	2422	2378 ± 111

Table 5.1. Main parameters (average values with relative standard deviation) collected from kinetic characterization tests of the III accumulation stage

	II stage (biomass selection) III Stage (PHA accumulation) and storage stage (quenching) of produced polymer					
Parameter	end of feast phase (II stage, SBR)	end of III stage	quencing 3 h (NaClO, 5% active Cl_2)	quencing 24 h (NaClO, 5% active Cl_2)	quencin 3 h (NaOH 1M)	quencin 24 h (NaOH 1M)
Biomass: *VSS (mg L^{-1})*	2286 ± 30	3486 ± 107	-	-	-	-
Active Biomass: X_A *(mg L^{-1})*	1871 ± 31	1770 ± 76	-	-	-	-
Total Suspended Solid *TSS (mg L^{-1})*	2739 ± 34	4044 ± 93	1644 ± 91	1425 ± 89	2494 ± 207	2200 ± 133
PHA *(mg L^{-1})*	415 ± 25	1716 ± 92	1520 ± 98	1378 ± 88	1331 ± 111	1212 ± 107
PHA *(mg d^{-1})*	415 ± 25	2059 ± 111	2127 ± 137	1929 ± 124	1863 ± 155	1697 ± 150
%PHA *(mg mg^{-1})*	18 ± 1	49 ± 2	90 ± 5	98 ± 5	54 ± 3	56 ± 5
TSS lowering *(%)*	-	-	52 ± 2	59 ± 2	29 ± 5	37 ± 4
PHA recovery *(%)*	-	-	106 ± 4	100 ± 5	87 ± 5	80 ± 6

Table 5.2. PHA, TSS-VSS comparison in SBR, accumulation and quenching stages from the 126th to 178th day (average values with relative standard deviation)

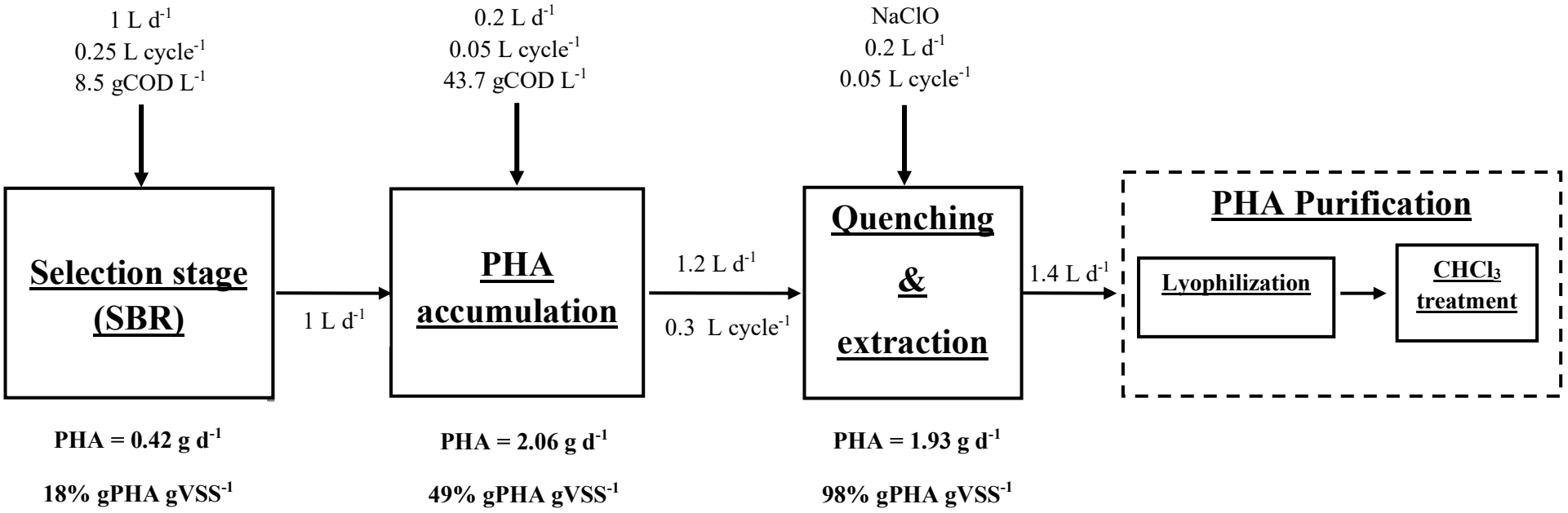


Figure 5.7. Scheme of the proposed process for PHA production (the first anaerobic stage is not included). The daily amount of PHA produced, the PHA content in the biomass, the flow rate of each step, the VFA concentration (COD) of each feeding solution are reported

CHAPTER 6. NUTRIENTS LEVEL EFFECT (N & P) ON A PHA ACCUMULATION CAPACITY OF ACCLIMATED BIOMASS

In addition to the internal limitation occurring under feast and famine conditions when sufficient growth nutrients are present, external limitations in form of nutrient limitation can increase the storage yield. In nutrient excess condition, the biomass growth usually plays an important role: specifically, Dionisi et al. (2005) observed, in typical batch tests, that the storage rate remained approximately constant at the beginning of the tests and then it increased slightly due to the simultaneous formation of the new active biomass. This new biomass was also able to store PHA; even though the storage played the major role in substrate removal during the entire tests, as the tests proceeded, the role of substrate oxidation and biomass growth became more important. In the same study, the authors observed a different biomass behaviour under nutrient limitation condition especially for the substrate removal rate which was lower. On the other hand, PHA concentration was similar in most of the different tests, indicating that external metabolic limitation did not increase storage rates. Furthermore, the storage rate under nutrient limitation tended to decrease (indicating the saturation of the biomass storage capacity), whereas it remained approximately constant in nutrient excess condition. However, under nutrient limitation, a higher fraction of the substrate is driven towards storage, occurring in a higher final PHA content. Consequently, under ammonia limiting conditions, the growth yield increased proportionally to the ammonia concentration while the storage yield decreases (Serafim et al. 2004). If the growth is allowed for a longer duration in batch operation, PHA content in the biomass will reach a maximum value from which it will decrease due to that growth becomes dominant over storage (Bengtsson et al. 2008).

Enrichment of a culture with a high storage potential is feasible both under nutrient excess (Bengtsson et al. 2008; Beccari et al. 1998; Takabatake et al. 2000) and under nutrient limitation (Lemos et al. 2006; Serafim et al. 2004). In addition to the organic carbon to nutrient levels, the nature of the organic substrate also plays a role. Selection of a PHA storing culture was not successfull applying the same organic

load rate and nutrient levels as Serafim et al. (2004) but with fermented molasses instead of synthetic acetate as substrate (Albuquerque et al. 2007). In this case excess nutrients were necessary for selecting a PHA storing culture. Excess nutrients during enrichment, therefore, result in higher biomass yield which positively affects the overall polymer productivity over the three-stage process.

Most of the previous studies, investigating different operating process condition (HRT, SRT, OLR, cycle length etc..) with mixed microbial cultures, explored a quite wider range on nitrogen levels than phosphorous levels in SBR and in batch accumulation tests. For cultures selected at very high growth rates, such as 1 day SRT in SBR fed with synthetic wastewater with nutrients excess (Dionisi et al., 2005 and 2006; Johnson et al., 2010), the overall process was feasible under different nitrogen levels imposed to the accumulation stage. Dionisi and co-authors (2005) observed that by increasing the nitrogen level in batch tests, the specific storage rate increased (from 208 to 649 $mgCOD_{PHA}$ $gCOD_{Xa}^{-1}$ h^{-1}), as well as the PHA productivity (from 0.08 to 0.37 gPHA L^{-1} h^{-1}). On the other hand, the final PHA content was higher under nitrogen limitation condition (61% gPHA $gVSS^{-1}$) than in excess (50% gPHA $gVSS^{-1}$). These results were confirmed by Johnson et al. (2010), even if the PHA productivity didn't follow the trend obtained by Dionisi et al. (2004, 2006). The higher PHA productivity and specific storage rate were obtained under nitrogen excess condition in batch. It is noteworthy to mention the very high PHA content obtained under nitrogen limitation (starvation) that was 89% gPHA $gVSS^{-1}$.

Bengtsson and co-workers (2008) investigated the PHA storage potential of a mixed culture selected in SBR under higher SRT (7 days), fed with fermented paper mill wastewater (PMW) in batch tests: different nitrogen and phosphorous levels were explored and the process was feasible in all conditions, in terms of storage rate and yields, final PHA content and PHA productivity.

A mixed culture growth on acetate in SBR at relatively high SRT (8 days) exhibited an higher PHA storage capacity at lower nitrogen level (Bertan Basak 2011); observed highest polymer yields for the biomasses enriched with and without nitrogen deficiency were 0.69 $gCOD_{PHA}$ $COD_{\Delta S}^{-1}$ and 0.51 $gCOD_{PHA}$ $COD_{\Delta S}^{-1}$, and corresponding polymer contents of biomasses were 43.3% ($gCOD_{PHA}$ $gCOD_{VSS}^{-1}$) and 38.3% ($gCOD_{PHA}$ $gCOD_{VSS}^{-1}$), respectively.

Wen and co-authors (2010) obtained a mixed culture in SBR at 10 days as SRT growth on acetate under nutrient excess. In the performed batch tests, different nitrogen and phosphorous levels were explored: the nitrogen limitation (by maintaining the phosphorous content in excess) improved the storage performance in terms of PHA content and specific storage rate; limiting ammonia availability during batch experiments also caused higher polymer productivity by suppressing biomass growth. The phosphorus level (by maintaining the same ammonia concentration) had less impact than nitrogen level effect: a hypothetical trend of biomass storage properties was not observed and an optimal phosphorus content seemed to be the best option.

PHA accumulation using fermented molasses was evaluated with two cultures selected either with acetate or fermented molasses in a study of Albuquerque et al. 2007. Storage yields varied from 0.37 to 0.50 Cmmol HA/Cmmol VFA. Low ammonia concentration (0.1 Nmmol/l) in the fermented molasses stimulated PHA storage (0.62 Cmmol HA/Cmmol VFA). In addition, strategies of reactor operation to select a PHA accumulating culture on fermented molasses were developed. The combination of low organic loading with high ammonia concentration selected a culture with a stable storage capacity and with a storage yield (0.59 Cmmol HA/Cmmol VFA) similar to that of the acetate-selected culture.

Pozo et al. (2011) evaluated the feasibility of PHA biosynthesis from kraft mill effluent using the batch system evaluating the biomass origin and C/N relationship influence. Two levels of BOD_5:N:P ratios (100:5:1 and 100:1:0.2) and three different sludge origins (sewage activated sludge, paper mill wastewater and kraft mill wastewater) were considered. The maximum PHA accumulation was obtained under a BOD5:N:P relationship of 100:1:0.2 on the third day of batch assays using PAS sludge with 25.7% of the cells accumulating PHA and on the fifth day in batch using SAS and KAS sludge with 25.9% and 30.4% of cells accumulating PHA, respectively.

Most of the studies on pure cultures PHA storage potential showed a relatively high phosphorus content in the culture medium, sometimes higher than nitrogen content (Qun Yan et al. 2010; Beom Soo Kim, 2000; Chakraborty et al. 2009; Aulenta et al.

2003), and higher than phosphorus content usually adopted in mixed microbial culture systems.

In a previous study of Chakraborty et al. (2009), a low cost medium to grow *Ralstonia eutropha* at high cell density was developed; the effects of different concentrations of VFAs on acid utilization rates, fermentation efficiency, PHA concentration and PHA productivity was determined. A carbon rich byproduct of corn ethanol production, condensed corn solubles (CCS), was used as an alternative medium. In addition to optimizing the CCS concentration, nitrogen was supplemented to maximize cell growth. When nitrogen became limiting at 48 h, VFAs (acetic, butyric, lactic and propionic acids) were added to aid PHA production. A CCS medium at concentration of 240 g L^{-1}, with a carbon:nitrogen ratio of 50:1 was developed as the optimal medium. Optimal levels of all the acids were determined to maximize PHA production. An overall comparison of the VFAs indicated that butyric and propionic acids were the most efficient carbon sources to maximize PHA production when added at the 5 g L^{-1} level.

In the Tables 6.1 and 6.2 the main results from previous studies, in terms of storage performance, are reported: in particular the nitrogen and phosphorus levels in both SBR and batch accumulation tests are highlighted; the composition of culture medium in some cases is also given.

The effects of nutrient levels on PHA accumulation were evaluated in tests performed with biomass enriched in PHA-storage organisms using a fermented diary effluent at AnoxKaldnes AB (Lund, Sweden).

	SBR			Accumulation									
	Substrate	*COD : N : P*	*SRT (d)*	*Substrate*	*COD : N : P*	COD/N (w/w)	COD/P (w/w)	%PHA (w/w)	Y_{STO} (COD/COD)	Y_{GROW} (COD/COD)	Y_{OBS} (COD/COD)	rPHA (mgCOD/gCOD/h)	PHA Productivity (gPHA/l/h)	MN (mg/L)
Wen et al., 2010	Acetate	100 : 5 : 0.5 (w)	10	Acetate	100 : 5 : 1 (w)	20	100	12	n.a.	n.a.	n.a.	70	0.16	(mg/L) 3.34 $CaCl_2 2H_2O$; 67 $MgSO_4$; 1.0 $FeCl_3 6H_2O$; 2.0 NaEDTA; 0.08 $ZnSO_4 7H_2O$; 0.06 $MnCl_2 4H_2O$; 0.1 H_3BO_3; 0.02 $CuSO_4$; 0.008 $NiCl_2 6H_2O$; 0.04 $NaMoO_4 2H_2O$
					100 : 5 : 0.63 (w)	20	160	17	n.a.	n.a.	n.a.	77	0.18	
					100 : 5 : 0.4 (w)	20	250	26	n.a.	n.a.	n.a.	140	0.34	
					100 : 5 : 0.2 (w)	20	500	17	n.a.	n.a.	n.a.	57	0.18	
					100 : 5 : 0.13 (w)	20	750	35	n.a.	n.a.	n.a.	89	0.2	
		100 : 2.5 : 1 (w)			100 : 5 : 1 (w)	20	100	12	n.a.	n.a.	n.a.	65	0.16	
					100 : 1.65 : 1 (w)	60	100	22	n.a.	n.a.	n.a.	103	0.29	
					100 : 1 : 1 (w)	100	100	41	n.a.	n.a.	n.a.	168	0.43	
					100 : 0.8 : 1 (w)	125	100	59	n.a.	n.a.	n.a.	255	0.78	
					100 : 0.5 : 1 (w)	180	100	45	n.a.	n.a.	n.a.	180	0.47	
Dionisi et al., 2006	Acetate Lactate Propionate (%; 40, 40, 20 COD basis)	100 : 4 : 1.4 (w)	1	Acetate Lactate Propionate (%; 40, 40, 20 COD basis)	100 : 4 : 5 (w)	25	10	n.a.	0.39 - 0.45	0.10 - 0.24	0.63 - 0.87	375 - 649	0.21 - 0.37	(mg/L) 50 $CaCl_2 2H_2O$; 100 $MgSO_4 7H_2O$; 2 $FeCl_3 6H_2O$; 3 NaEDTA; 0.01 $ZnSO_4 7H_2O$; 0.03 $MnCl_2 4H_2O$; 0.3 H_3BO_3; 0.2 $CoCl_2 6H_2O$; 0.02 $NiCl_2 6H_2O$; 0.01 $CuCl_2 2H_2O$; 0.03 $NaMoO_4 2H_2O$; 20 Thiourea
		100 : 6.6 : 1.2 (w)			100 : 4 : 13.3 (w)	25	7.5	n.a.	0.39	0.2	0.77	278	0.16	
		100 : 4.2 : 0.9 (w)			100 : 4 : 15 (w)	25	6.7	n.a.	0.46	0.15	0.71	392	0.22	
		100 : 5 : 0.9 (w)			100 : 4 : 18 (w)	25	5.5	n.a.	0.1	0.34	0.57	44	0.03	
		100 : 4 : 0.7 (w)			100 : 4 : 18 (w)	25	5.5	n.a.	0.05	0.4	0.56	22	0.01	
Dionisi et al., 2005	Acetate Lactate Propionate (%; 40, 40, 20 COD basis)	100 : 6.6 : 1.2 (w)	1	Acetate Lactate Propionate (%; 40, 40, 20 COD basis)	100 : 3.3 : 10 (w)	30	10	~50	0.39	0.2	0.77	278	0.16	(mg/L) 50 $CaCl_2 2H_2O$; 100 $MgSO_4 7H_2O$; 2 $FeCl_3 6H_2O$; 3 NaEDTA; 0.1 $ZnSO_4 7H_2O$; 0.03 $MnCl_2 4H_2O$; 0.3 H_3BO_3; 0.2 $CoCl_2 6H_2O$; 0.02 $NiCl_2 6H_2O$; 0.01 $CuCl_2 2H_2O$; 0.03 $NaMoO_4 2H_2O$; 20 Thiourea
					100 : 0 : 10 (w)	infinity	10	61	0.31	n.a.	n.a.	208	0.08	

	SBR			Accumulation									
	Substrate	*COD : N : P*	*SRT (d)*	*Substrate*	*COD : N : P*	COD/N (w/w)	COD/P (w/w)	%PHA (w/w)	Y_{STO} (COD/COD)	Y_{GROW} (COD/COD)	Y_{OBS} (COD/COD)	rPHA (mgCOD/gCOD/h)	PHA Productivity (gPHA/l/h)	MN (mg/L)
Basak et al., 2011	Acetate	100 : 12 : 2 (w)	8	Acetate	100 : 12 : 2 (w)	8	50	38	0.51	n.a.	n.a.	240	0.61	3mg/L $FeSO_4$; 1.5mg/L $ZnSO_47H2O$; 8mg/L $CaCl_22H_2O$; 1.2mg/L $MnSO_4H2O$; 45mg/L $MgSO_47H_2O$;
		100 : 2 : 2 (w)			100 : 2 : 2 (w)	50	50	43	0.69	n.a.	n.a.	340	0.43	
Albuquerque et al., 2007	Acetate	n.a.	10	Sugar Cane Molasses	100 : 7.4 : - (mol)	13.5 (mol)	n.a.	n.a.	0.45	0.37	n.a.	0.20 mol/molX_A/h	0.62	Thiourea and MN solution (not specified)
					100 : 7 : - (mol)	14 (mol)	n.a.	n.a.	0.37	0.34	n.a.	0.28 mol/molX_A/h	0.67	
					100 : 4.7 : - (mol)	21 (mol)	n.a.	n.a.	0.5	0.23	n.a.	0.23 mol/molX_A/h	0.83	
					100 : 0.09 : - (mol)	1090 (mol)	n.a.	n.a.	0.62	0.004	n.a.	0.23 mol/molX_A/h	0.96	
				Synthetic Mixture VFAs	100 : 0.08 : - (mol)	1200 (mol)	n.a.	n.a.	0.62	0.004	n.a.	0.37 mol/molX_A/h	0.86	
	Sugar Cane Molasses	n.a.		Sugar Cane Molasses	100 : 1 : - (mol)	100 (mol)	n.a.	n.a.	0.44	0.05	n.a.	0.12 mol/molX_A/h	0.42	
				Sugar Cane Molasses	100 : 0.2 : - (mol)	610 (mol)	n.a.	n.a.	0.59	0.01	n.a.	0.14 mol/molX_A/h	0.59	
Johnson et al., 2010	Acetate	100 : 7 : 2 (w)	1	Acetate	n.a.	infinity	n.a.	89	n.a.	n.a.	n.a.	2.10 mol/molX/h	0.15	1.25 mM KH_2PO_4; 0.3mM $MgSO_47H2O$; 0.35 mM KCl; 5 mg/L Thiourea
					n.a.	40	n.a.	77	n.a.	n.a.	n.a.	3.00 mol/molX/h	0.11	
					n.a.	8	n.a.	69	n.a.	n.a.	n.a.	3.30 mol/molX/h	0.2	
Albuquerque et al., 2010	Sugar Cane Molasses	100 : 3 : 1 (w)	10	Sugar Cane Molasses	100 : 0.16 : - (mol)	600 (mol)	n.a.	33	0.68	n.a.	n.a.	0.30 mol/molX/h	0.13	Thiourea and MN solution (not specified)
					100 : 0.2 : - (mol)	500 (mol)	n.a.	44	0.84	n.a.	n.a.	0.15 mol/molX/h	0.21	
					100 : 0.5 : - (mol)	2000 (mol)	n.a.	44	0.68	n.a.	n.a.	n.a.	0.21	
					100 : 0.1 : - (mol)	1000 (mol)	n.a.	45	0.71	n.a.	n.a.	0.24 mol/molX/h	0.25	
					100 : 0.15 : - (mol)	600 (mol)	n.a.	61	0.67	n.a.	n.a.	0.21 mol/molX/h	0.46	
					100 : 0.03 : - (mol)	3000 (mol)	n.a.	61	0.71	n.a.	n.a.	n.a.	0.46	
					100 : 0.08 : - (mol)	1200 (mol)	n.a.	55	0.66	n.a.	n.a.	0.13 mol/molX/h	0.37	
Mengmeng et al., 2009	n.a.	n.a.	n.a.	Excess Sludge Fermented	100 : 3 : 0.4 (w)	34	234	56.5	n.a.	n.a.	n.a.	310	0.31	n.a.

	SBR			Accumulation									
	Substrate	*COD : N : P*	*SRT (d)*	*Substrate*	*COD : N : P*	COD/N (w/w)	COD/P (w/w)	%PHA (w/w)	Y_{STO} (COD/COD)	Y_{GROW} (COD/COD)	Y_{OBS} (COD/COD)	rPHA (mgCOD/gCOD/h)	PHA Productivity (gPHA/l/h)	MN (mg/L)
Bengtsson et al., 2008	n.a.	n.a.	7	Paper Mill Wastewater	100 : 0.03 : 0.0015 (w)	3300	66670	42.7	0.67	0.26	n.a.	0.046 mol/molX/h	0.09	n.a.
					100 : 0.03 : 1.9 (w)	3300	50	44	0.55	0.13	n.a.	0.056 mol/molX/h	0.13	
					100 : 4.7 : 0.0015 (w)	21	66670	48.2	0.66	0.18	n.a.	0.064 mol/molX/h	0.12	
					100 : 4.7 : 1.9 (w)	21	50	31.9	0.33	0.31	n.a.	0.056 mol/molX/h	0.12	
Johnson et al., 2009	Acetate	100 : 9 : 1.2 (w)	4	Acetate	100 : 0 : 1.2 (w)	infinity	83.3	60	n.a.	n.a.	n.a.	n.a.	n.a.	8.3 mM KH_2PO_4; 1.85mM $MgSO_4 7H_2O$; 2.4 mM KCl; 5 mg/L Thiourea
		100 : 5 : 1.2 (w)			100 : 0 : 1.2 (w)	infinity	83.3	70	n.a.	n.a.	n.a.	0.12 mol/molX/h	n.a.	
		100 : 4 : 0.6 (w)			100 : 0 : 0.6 (w)	infinity	167	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
		100 : 2 : 0.6 (w)			100 : 0 : 0.6 (w)	infinity	167	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
		100 : 4 : 1.2 (w)	1		100 : 0 : 1.2 (w)	infinity	83.3	60	n.a.	n.a.	n.a.	n.a.	n.a.	
		100 : 4 : 0.1 (w)			100 : 0 : 0.1 (w)	infinity	1000	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
		100 : 5 : 0.1 (w)	0.5		100 : 0 : 0.1 (w)	infinity	1000	70	n.a.	n.a.	n.a.	n.a.	n.a.	
Reddy and Mohan. 2012	Glucose	100 : 4 : 3 (w)	n.a.	Glucose	100 : 2.2 : 2.2 (w)	45.4	45.4	45.1	n.a.	n.a.	n.a.	n.a.	0.02	(mg/L) 300 $MgCl_2$; 25 $CoCl_2$; 11.5 $ZnCl_2$; 10.5 $CuCl_2$; 5 $CaCl_2$; 15 $MnCl_2$; 16 $NiSO_4$; 25 $FeCl_3$;
					100 : 4.4 : 2.2 (w)	22.7	45.4	41.5	n.a.	n.a.	n.a.	n.a.	0.02	
					100 : 6.6 : 2.2 (w)	15.1	45.4	38	n.a.	n.a.	n.a.	n.a.	0.02	
					100 : 2.2 : 1.1 (w)	45.4	90.8	54.2	n.a.	n.a.	n.a.	n.a.	0.03	
					100 : 2.2 : 2.2 (w)	45.4	45.4	49.3	n.a.	n.a.	n.a.	n.a.	0.02	
					100 : 2.2 : 3.3 (w)	45.4	30.3	45.5	n.a.	n.a.	n.a.	n.a.	0.02	
Pozo et al., 2011	Kraft mill wastewater	n.a.	n.a.	Kraft mill wastewater	100 : 1 : 0.2 (w)*	100*	500*	30.4	0.14	0.47	n.a.	4.6	0.023	n.a.
	Sewage wastewater	n.a.	n.a.			100*	500*	25.9	0.11	0.42	n.a.	3	0.015	
	Paper mill wastewater	n.a.	n.a.			100*	500*	25.7	0.1	0.39	n.a.	3.44	0.017	

Table 6.1. Cultivation conditions (SBR) and accumulation strategies (batch reactor) for PHA production from mixed microbial cultures by using real and synthetic feed solutions with different nutrient levels. The PHA content was calculated as gram of PHA per gram of VSS.
n.a.: Not available; w: weight (ratio calculated on gram basis); mol: molar basis
* relationship in terms of BOD_5:N:P

		Accumulation										
		Substrate	*COD : N : P*	COD/N (w/w)	COD/P (w/w)	%PHA (w/w)	Y_{STO} (COD/COD)	Y_{GROW} (COD/COD)	Y_{OBS} (COD/COD)	rPHA (mgCOD/gCOD/h)	PHA Productivity (gPHA/l/h)	MN (mg/L)
Yan et al., 2010	*Bacillus cereus*	Acetic, Propionic, Butyric and Lactic Acid	100 : 15 : 20 (w)	6.7	5	44	0.4	0.47	n.a.	45	0.09	(g/L) 120 $MgSO_4$; 1.7 citric acid; 2.25 $ZnSO_4 7H_2O$; 10 $FeSO_4 7H_2O$; 2.25 $CuSO_4 5H_2O$; 0.5 $MnSO_4 5H_2O$; 0.02 $CuSO_4$; 2 $CuCl_2 2H_2O$; 0.1 $NaMoO_4 2H_2O$; 0.3 H_3BO_3
Mohd et al., 2009	*Comamonas* EP 172	Butyric Acid	100 : 10 : 10 (w)	10	10	36	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			100 : 5 : 10 (w)	20	10	32	n.a.	n.a.	n.a.	n.a.	n.a.	
			100 : 3.33 : 10 (w)	30	10	46	n.a.	n.a.	n.a.	n.a.	n.a.	
			100 : 2.5 : 10 (w)	40	10	39	n.a.	n.a.	n.a.	n.a.	n.a.	
			100 : 2 : 10 (w)	50	10	40	n.a.	n.a.	n.a.	n.a.	n.a.	
		Anaerobically treated POME (VFAs mixture)	100 : 2 : 10 (w)	50	10	59	0.48	0.45	n.a.	20	0.18	
Aulenta et al., 2003	*Amaricoccus kaplicensis*	Acetate	100 : 7 : 13.3 (w)	14.3	7.5	n.a.	0.65	0.06	0.76	78	0.23	0.002g/L $FeCl_3$; 0.003g/L EDTA; 0.05g/L $CaCl_2 2H_2O$; 0.1g/L $MgSO_4 7H_2O$; Vitamin mix
			100 : 7.8 : 15 (w)	12.7	6.7	27	0.61	0.09	0.77	68	0.2	
			100 : 10.9 : 20.8 (w)	9.2	4.8	n.a.	0.71	0.04	0.75	62	0.19	
			100 : 20 : 38 (w)	5	2.6	n.a.	0.56	0.03	0.77	100	0.3	
Kim, 2000	*Azotobacter chroococcum*	Starch	100 : 0.84 : 1.15 (w)	119	87	46	0.25	0.54	n.a.	16	0.35	0.4g/L $MgSO_4 7H_2O$; 1g/L citric acid; Trace elements
	Escherichia coli	Whey	100 : 0.84 : 3 (w)	119	33.3	80	0.56	0.31	n.a.	77	0.48	1.2g/L $MgSO_4 7H_2O$; 1.7g/L citric acid; Trace elements
Chakraborty et al., 2009	*Ralstonia eutropha*	Acetic Acid	100 : 2 : 3.5 (w)	50	28.6	29.2	0.48	n.a.	n.a.	12	0.024	n.a.
		Butyric Acid				31.9	0.77	n.a.	n.a.	13.2	0.037	
		Lactic Acid				40.7	0.4	n.a.	n.a.	16.7	0.02	
		Propionic Acid				29.3	0.72	n.a.	n.a.	12.2	0.036	

Table 6.2. Cultivation conditions strategies (batch accumulation) for PHA production from pure cultures by using real and synthetic feed solutions with different nutrient levels. The PHA content was calculated as gram of PHA per gram of VSS. n.a.: Not available; w: weight (ratio calculated on gram basis)

6.1 Materials and methods

6.1.1 Batch tests (PHA accumulation)

Setup

Biomass that was used in the experiments was enriched with a fermented diary wastewater to produce activated sludge with high PHA accumulating potential. Batch tests were carried out in a 500 mL working volume reactor at the same temperature (30°C) and pH (~8.5) as the SBR. The different types of test were defined based on the concentration level of nitrogen and phosphorous in the synthetic feed, consisting of acetic acid in average concentration of 50 g L^{-1} on a COD basis, NH_4Cl and KH_2PO_4 in different amount based on the chosen range of COD/N and COD/P ratios. The experimental plan was focused on the effects of different COD:N:P ratios (from starvation to excess of these nutrients level) on the storage performance of the biomass; for each type of conditions, a reference batch test was made using a different feed, containing a fixed nitrogen and phosphorous concentration, theoretically defined as balance nutrients condition (COD:N:P = 100:1:0.9). Thus, the accumulation tests were conducted in 2 parallel reactors under aerobic conditions; each single batch test was named based on the total nutrients consumption (nitrogen and phosphorous).

The air was supplied by an air pump through a glass diffuser, the temperature was controlled via water jackets connected to water baths, and mixing was magnetically provided (400 rpm). Air flow rates, DO levels, pH and temperature were monitored. DO levels were maintained in a range 1 - 4.5 mg L^{-1} to avoid oxygen limitation during the batch tests. The mixed liquor was first aerated until constant pH and DO levels were achieved (almost 20 minutes). Substrate feed aliquots were dosed at fixed volumes via a diaphragm pump (Grundfos DME) targeting a maximum substrate concentration (0.2 gCOD L^{-1}) based on a multiple-spiking respirometric control approach. This value was determined by the initial biomass concentration in batch reactor; it was chosen a fixed value for all the tests approximately equal to 0.1$gCOD_{Substrate}$ $gTSS^{-1}$.

Procedures

For all the experiments, the biomass was sampled from SBR at the end of the cycle, centrifuged and suspended in a synthetic buffer solution that helped to reset pH changes after addition of acid feed and retain the buffering capacity of the sludge. Based on the suspended solid concentration (SS) in the SBR, the biomass was concentrated after centrifugation up to ~2 gTSS L^{-1}. The composition of the buffer solution was (g L^{-1}): Na_2CO_3 (0.248), $NaHCO_3$ (0.262), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.25). For each liter of concentrated biomass was needed, 0.8L of buffer solution was used to resuspend it.

Other necessary trace elements were provide to the rinsed biomass, before starting the accumulation tests, by single addition of few mL from two different stock solutions (the Iron-Zinc solution and the Trace elements solution) directly into the batch reactors. From the Iron-Zinc solution and from the Trace elements solution were taken 0.2 mL and 0.6 mL respectively for 0.5 L of reactor. The composition of the Iron-Zinc solution was (g L^{-1}): $FeCl_3 \cdot 6H_2O$ (7.8), $ZnSO_4 \cdot 7H_2O$ (0.78); the composition of the Trace elements solution B (g L^{-1}): H_3BO_3 (0.25), $CoCl_2 \cdot 6H_2O$ (0.25), $MnCl_2 \cdot 2H_2O$ (0.205), $NaMoO_4 \cdot 2H_2O$ (0.1), $CuSO_4 \cdot 5H_2O$ (0.05), KI (0.3).

The pH of the feed was adjusted to 3.5 with 4M NaOH. The accumulation tests were performed for 24 hours. During each test, between 4 - 10 ml from both reactors were collected at almost regular intervals until 12 hours and the final sampling at 24 hours, for PHB and polysaccharide analysis, total soluble COD, nitrogen and phosphorous quantification. The liquid sludge samples were centrifuged (3500 rpm for 5 minutes) and from the supernatant, a measurements of soluble COD, nitrogen and phosphorous were done after filtration (analytical techniques). The pellets were dried at 70°C for one night, weighed for TSS measurements and used for PHB and polysaccharide analysis. An aliquot of initial and final sample of biomass (1-2 mL) was also taken in order to determinate the total nitrogen and phosphorous content in the biomass. The TSS and VSS concentration were also determined at the beginning of the tests (10 mL from rinsed biomass), in the middle and at the end of the tests (5mL) based on standard methods (APHA, 1998).

6.1.2 Analytical Techniques

Soluble compounds

Process performance during PHB accumulation tests were evaluated by measuring COD uptake, PHB specific production rate, storage yield, nutrient consumption and solid levels in the time, until the end of the tests. A second sample was centrifuged and filtered through a Munktell MGA micro glass fibre paper (1.6 μm) and the filtrate was analyzed with Hach-Lange cuvette kits for soluble COD (COD_{sol}, kit # LCK114), N-NH_4^+ (LCK 138 and 238) and P-PO_4^{3-} (LCK 349 and 350). A Hach-Lange LT100 incubation block and a Xion 500 Hach-Lange spectrophotometer were used. Proper dilutions were conducted with distilled water. Temperature, DO, pH (pH/Oxi 340i, WTW; Endress + Hauser Liquisys M, pH 4 and 7, Reagecon standards) were monitored inside the reactors.

PHB and Polysaccharides

Calibrations for the PHB response factor by GC–flame ionization detector (FID) during the method development were achieved with poly-3-hydroxybutyrate (PHB) standard (Sigma-Aldrich). This standard was weighed directly into the glass tubes, as different aliquots in the range 2-12 mg. D-Glucose (Sigma-Aldrich) was used as the standard for cellular carbohydrate content. Glucose standard was weighed directly into the glass tubes as above for PHB, in the range 2-10 mg. Extracted long chain microbial fatty acids were calibrated with respect to hexadecanoic acid, C16 (Sigma-Aldrich). Aliquots of C16 prepared as a stock solution in hexane were dispensed into the glass tubes and processed as above.

Benzoic acid (Riedel-de-Haën, 99.9%) was used as the method reference standards (RS) for calibration. The benzoic acid stock solution was prepared in butanol at 10 mg mL^{-1}. Extraction standards (ES) and internal standards (IS) were also used during the method development. Stock solutions of methyl 3-hydroxybutyrate (Sigma-Aldrich) and selected esters of saturated fatty acids (methyl esters of C8–C22, Sigma-Aldrich) were prepared as stock solutions in hexane. All solvents used were Merck LiChrosolvs grade. Aqueous stock solutions were prepared with deionized water. All glassware was cleaned and either hexane rinsed or oven fired at 425°C prior to use.

The PHB in the biomass was subjected to acid alcoholysis (butanol and HCl) followed by hexane extraction. P(3HB) and Glucose (Aldrich 36,350-2) were used as standards for 3HB and polysaccharides, respectively. After extraction, the organic phase was analyzed in a Varian 3800 GC equipped with 2 parallel channels with FID and a Saturn 2000 GM/MS/MS detector (VF-1 ms columns 30 m x 0.25 mm, 1 μm thickness).

Sample processing for GC/FID and/or GC/MS analysis

For PHB derivatization, 1.5 mL of normal butyl alcohol (or propanol) and 0.5mL concentrated hydrochloric acid (Merck Proanalysi, 37%) were added in the glass tubes to the dried biomass samples and dried calibration standards. A 50 μL aliquot of RS was subsequently added to all tubes as a method control and volume correction standard. The tubes were capped and incubated at 100°C for 8h and subsequently cooled back to room temperature. Shorter and longer incubation times were used during the method development. After cooling, 2.5 mL hexane, and 4 mL deionized water were added. In some cases, a FAME extraction standard was added (50 μL from a stock solution in hexane). The tubes were vortex mixed (for 10 seconds). After phase separation, 3.5 mL of the underlying aqueous phase were removed and discarded. A second 4 mL aliquot of deionized water was added in order to rinse the solvent phase of acid. The test tubes were then vortex mixed (10 seconds), and centrifuged at 3000 g for 20 minutes, in order to remove any solid debris from the upper solvent phase. One milliliter of the upper solvent phase was transferred directly to standard 2 mL GC vials.

GC/FID and GC/MS analyses

A Varian 3800 Series gas chromatograph, equipped with a Varian 8400 autosampler, a FID, and a Saturn 2000 GC/MS/MS detector, was used. For these analyses, the best chromatography for the HB esters was obtained with Varian VF-1ms columns (30mx0.25mm, 1.00 μm film thickness). Injection temperatures of 250 1C [CP-1177 (FID) and CP-1079 (MS) injectors] were applied with standard single gooseneck split/splitless inserts. The oven program following 1 μL (FID) and 0.3 μL (MS) injections was as follows: 5min hold at 70°C, 10°C/min to 290°C, and hold for 18

min. Split injections were made with 20:1 (FID) and 25:1 (MS). Injection chambers were purged after 0.5 and 1 min for the FID and MS columns, respectively. A helium carrier gas with constant flow control was used with 1 (FID) and 0.7 (MS) mL/min. The FID detector temperature was 300°C. MS analysis was for fragments from 40 to 450m/z produced with EI auto ionization.

6.1.3 Calculations

The PHB content in the biomass was calculated as the fraction of g PHB per g VSS. PHA yields were calculated by dividing the amount of PHB formed (as COD by using the conversion factor 1.674 gCOD $gPHB^{-1}$) by either the total COD substrate consumed (assumed equal to total acetic acid consumed; $Y_{P/\Delta S}$ on a COD basis). Other yields were determined based on the amount of glucose formed (as COD; 1.067 gCOD $gGlu^{-1}$) per total COD substrate consumed for the yield of polysaccharides ($Y_{PS/\Delta S}$); and based on the amount of the active biomass formed per total COD substrate consumed for the growth yield ($Y_{Xa/\Delta S}$). The active biomass (X_A) was estimated by subtracting the amount of PHB from the VSS values. A standard composition of the biomass, $C_5H_7NO_2$, was used; thus, a conversion factor of 1.42 gCOD g X_A was considered for the calculations of the yield and specific rate, both estimated on a COD basis. The specific PHB production rates (q_P, mgPHB gX_A^{-1} h^{-1}) and specific COD substrate uptake rates ($-q_S$, $mgCOD_S$ $gCOD_{Xa}^{-1}$ h^{-1}) were calculated based on the evaluation of a regression analysis. Two functions were taken into account in order to plot PHB, polysaccharides and solid (TSS, VSS, X_A) mass experimental data (sigmoid [1] and exponential [2] functions):

$$[1]\ y = \alpha + \frac{\beta}{1+ e^{-(x-x0)k}}$$

$$[2]\ y = \alpha + \beta\,(1 - e^{-kx})$$

The chosen function based on the minimizing squared differences, was derivative in order to quantify the kinetics of substrate degradation and PHB storage. It is important to notice that, the storage and substrate uptake specific rates were calculated based on the initial X_A.

CHAPTER 7. CONCLUSIONS

The quickness of the activated sludge acclimation to the newly imposed feast and famine regime (in SBR) and the effects of acclimation period on storage performance was investigated.

A short feast phase length during the SBR cycle (less than 20% of the cycle length) was the key parameter to obtain good selective pressure on PHA-storing microorganisms and consequently PHA storage at high rate and yield. Even if this condition was reached quickly (in no more than 3.33 HRT, it was not steadily maintained and a highly unstable SBR performance was observed for at least 8-10 days. However, by sampling the biomass from SBR cycle characterized by short feast phase, the storage response in the accumulation stage was similar to that obtained with fully acclimated biomass (characterized by a longer acclimation period), despite of the partial acclimation. Cluster analysis of DGGE profiles relative to the long-term SBR run, indicated that feast and famine conditions were anyway able to steadily select for PHA-storing microbial species with high PHA storage capabilities, in spite of small changes of microbial structure.

This evidence suggests a new strategy for operating the overall PHA production in a single reactor for biomass selection/enrichment and PHA accumulation. In this novel approach, the first achievement of a short feast phase in SBR can be easily verified by monitoring of the dissolved oxygen profile. As soon as the short feast phase has been reached, the PHA accumulation stage is directly performed in the SBR, by feeding the biomass at a higher substrate load. Based on present experimental data, the best PHA productivity was around 0.85 gPHA L^{-1} d^{-1} and final PHA content of 0.48 COD COD^{-1}. Even though this value is lower than best PHA productivity achievable by using the more documented approach (1.6 ± 0.1 gPHA L^{-1} d^{-1}, Beccari et al. 2010), the proposed approach is anyway attractive because of its simplicity (a single reactor instead of two ones), also considering that this single reactor performance can be certainly optimized by investigating the SBR start up in a wider range of typical operating parameters (e.g. OLR, cycle length).

By maintaining the same OLR (8.5 gCOD L^{-1} d^{-1}) and HRT (1 d), the more traditional approach was investigated by changing the overall cycle length in a range

2-8 h. The decrease of the overall SBR cycle length determined an increase in polymer production rates. It appeared clearly that until a cycle length of 6 h (feed frequency 4 times per day), the process was feasible at relatively high OLR. At 8 h of cycle length, the biomass storage response was not appropriate; furthermore, an increasing of feeding time led to a decrease of the maximum substrate concentration at which the biomass was exposed and in a drastic drop of the storage capacity. These concepts were easily explained by considering the importance of feast phase (Beccari et al. 1998; Dionisi et al. 2004; Serafim et al. 2004; Dionisi et al. 2005b; Dionisi et al. 2006; Dionisi et al. 2007; Johnson et al. 2009; Albuquerque et al. 2010; Beccari et al. 2010; Bengtsson et al. 2010; Jiang et al. 2011; Valentino et al. 2012): with a feast phase percentage around 20%, the process was sustainable; its sustainability was compromised for higher values. It is also noteworthy, that during the Run II (cycle 6 h) and III (cycle 2 h), similar performance was obtained even with similarity of the structure of the microbial consortium. The DGGE profiles showed that Lampropedia hyalina genus (recently detected as the dominant microorganisms in PHA producing SBR, Beccari et al. 2009; Beccari et al. 2010) was stably enriched in both runs where the highest storage response was observed. A phylotype closely related to Meganema perideroedes and Thauera-related phylotype were detected in Run I (cycle 8 h, which presence has been observed in SBR for PHA production, Dionisi et al. 2005b; Dionisi et al. 2006; Beccari et al. 2009); shortening the feeding time (to 10 min) induced the enrichment of a Hydrogenophaga sp. strain and Thauera phylotype, the most prominent community member at the end of the Run.

The PHA accumulation performance confirmed the importance to carry out the selection stage in a better way, for the choices of cycle length and feeding time. In fact, the polymer productivity in the three-stage process (which depends on both biomass concentration and polymer production rate, Dionisi et al. 2005b; Dionisi et al. 2006; Dionisi et al. 2007), reached a maximum value of 1.71 gPHA L^{-1} d^{-1} at an overall cycle length of 2 h sampling the biomass at the end of feast phase; despite of the higher biomass concentration in the SBR run carried out with the overall cycle length of 8 h.

However, in a perspective of selection and PHA accumulation stages implemented continuously in a larger scale, setting the SBR cycle length at 6 h (and discharging the biomass 4 times per day) could simplify the process management, despite of the lower PHA productivity (1.25 gPHA L^{-1} d^{-1}): in fact, a time of 6 h was more than enough in order to reach a PHA biomass saturation, and no starvation time is necessary from the II and III stage. On the other hand, the SBR cycle length of 2 h assured the best storage performance in terms of PHA productivity (the final PHA content was similar in Run II and III after batch tests); however this storage reponse could be partially lost by setting a starvation time before to begin the accumulation stage, as demonstrated by the short-term batch tests.

Thus, a cycle length of 6 h was chosen for the implementation of the II and III stage process in a continuous way. The imposed feast and famine regime occurred in a good storage response; the feast phase percentage was 20.4 ± 0.6 %, the specific storage rate was 179 ± 8 mgCOD gCODnonPolym^{-1} h^{-1}, the storage yield was 0.27 ± 0.01 COD COD^{-1}. The III stage was implemented with the same time of 6 h, that was enough to reach polymer saturation of the biomass, directly loaded from the SBR without any dilution. It should be taken into account that a different and more concentrated than SBR synthetic feed was used (43.66 $mgCOD_{TOT}$ L^{-1}), and this could be not feasible in the perspective of a large-scale process. On average, the amount of PHA produced at the end of III stage was 2059 ± 111 mg d^{-1} (5 times higher than value of II stage), at 50% as content in the biomass (gPHA $gVSS^{-1}$). For the extraction and purification steps, also implemented in continuous way, the 24 h NaClO (5% active Cl_2) treatment involved in a high TSS lowering (59%) and PHA recovery (~100%), that occurred in a further increasing of the PHA content (98% gPHA $gVSS^{-1}$). The solid matter was then lyophilized and purified via $CHCl_3$ treatment: in terms of composition, the final HV percentage decreased from 9.40% to 7.74% gHV $gPHA^{-1}$ in the lyophilized samples and in the films respectively.

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