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Impact of carbon/nitrogen feeding strategy on polyhydroxyalkanoates production using mixed microbial cultures

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Dezembro de 2015

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Acknowledgments

Firstly, I would like to thank both Dr. Maria Ascensão Reis and Dr. Mauro Majone, for providing me the fantastic opportunity to work on this project which would have not been possible without you.

I am also grateful to Dr. Marianna Villano, Sabrina Campanari and Stefania Matteo for the scientific guidance, welcoming and supporting me during my stay. Besides, I would like to acknowledge all other members of the Lab 026, for their kind support and friendship.

I would like to thank my dear parents and my beloved girlfriend, Rita, to whom I dedicate this thesis. I'm forever grateful to you, for your infinitive support and love and for always being there for me.

I am also grateful to all my friends for all the support and good moments shared over the years.

The year spent in Rome was unique and memorable. It was quite an experience which allowed me to grow and learn so much. Thank you very much Italy, I hope to be back some day.

Resumo

A produção de polihidroxialcanoatos (PHAs) usando culturas microbianas mistas (MMC) requer um processo *multi-stage*, envolvendo a selecção de organismos acumuladores de PHA, e é frequentemente feito num reactor descontínuo sequencial (SBR). Como os *feedstocks* renováveis de baixo custo geralmente têm um baixo conteúdo de azoto, a adição de azoto é necessária durante a fase de selecção para promover o crescimento celular. Assim sendo, foi avaliada nesta dissertação a possibilidade de alimentar as fontes de carbono e azoto separadamente. Para além disso, três rácios COD:N distintos (100:3.79, 100:3.03 e 100:2.43) foram testados, permitindo a análise dos mesmos na performance do SBR.

Para cada reactor, uma mistura de ácidos acético e propiónico com uma carga orgânica de 8.5 gCOD L^{-1} d^{-1} foi usada como fonte de carbono, e o sulfato de amónio foi usado como fonte azoto num SBR à escala laboratorial, com um volume de 1 L – o tempo de retenção das lamas foi de 1 d, e a duração do ciclo de 6 h. A estratégia de alimentação estudada resultou numa melhoria significativa da pressão selectiva para organismos acumuladores de PHA, bem como na duplicação de PHA produzido (até cerca de 1.3 gCOD L^{-1}). Uma resposta de acumulação elevada foi observada nos dois reactores com COD:N mais reduzido, enquanto o rácio mais elevado resultou numa fraca prestação em termos de produção de polímero. De facto, a forte limitação de azoto levou ao crescimento de fungos, e a uma baixa capacidade de armazenamento pelos microorganismos presentes. O rácio de COD:N afectou também a composição do polímero, pois o poli(3-hidroxibutirato-co-3-hidroxivalerato) (PHBV) produzido, apresentou um conteúdo de HV variável (1-20 %, w/w) nos três reactores, sendo menor para rácios de COD:N maiores. Este resultado sugere a possibilidade de usar o rácio COD:N como ferramenta para alterar o conteúdo de HV, sem no entanto alterar a composição do *feedstock.*

Palavras-chave: Ácidos gordos voláteis, culturas microbianas mistas, polihidroxialcanoatos, rácio COD:N, reactor descontinuo sequencial, regime de fome e fartura

Abstract

Polyhydroxyalkanoates (PHA) production using mixed microbial cultures (MMC) requires a multi-stage process involving the microbial selection of PHA-storing microorganisms, typically operated in sequencing batch reactors (SBR), and an accumulation reactor. Since low-cost renewable feedstocks used as process feedstock are often nitrogen-deficient, nutrient supply in the selection stage is required to allow for microbial growth. In this context, the possibility to uncouple nitrogen supply from carbon feeding within the SBR cycle has been investigated in this study. Moreover, three different COD:N ratios (100:3.79, 100:3.03 and 100:2.43) were tested in three different runs which also allowed the study of COD:N ratio on the SBR performance.

For each run, a synthetic mixture of acetic and propionic acids at an overall organic load rate of 8.5 gCOD L^{-1} d^{-1} was used as carbon feedstock, whereas ammonium sulfate was the nitrogen source in a lab-scale sequence batch reactor (SBR) with 1 L of working volume. Besides, a sludge retention time (SRT) of 1 d was used as well as a 6 h cycle length. The uncoupled feeding strategy significantly enhanced the selective pressure towards PHA-storing microorganisms, resulting in a two-fold increase in the PHA production (up to about 1.3 gCOD L^{-1}). A high storage response was observed for the two runs with the COD:N ratios (gCOD:gN) of 100:3.79 and 100:3.03, whereas the lowest investigated nitrogen load resulted in very poor performance in terms of polymer production. In fact, strong nitrogen limitation caused fungi to grow and a very poor storage ability by microorganisms that thrived in those conditions. The COD:N ratio also affected the polymer composition, indeed the produced poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) showed a variable HV content (1-20 %, w/w) among the three runs, lessening as the COD:N increased. This clearly suggests the possibility to use the COD:N ratio as a tool for tuning polymer properties regardless the composition of the feedstock.

Keywords: COD:N ratio, feast and famine regime, mixed microbial cultures, polyhydroxyalkanoates, sequential batch reactor, volatile fatty acids

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List of Abbreviations

3-HDD	3-hydroxydodecanoate
3-HO	3-hydroxyoctanoate
4-HA	4-hydroxyalkanoate
5-HA	5-hydroxyalkanoate
Acetyl-CoA	Acetyl Coenzyme A
Acyl-CoA	Acyl Coenzyme A
ADF	Aerobic dynamic feeding
APME	Association of Plastics Manufactures in Europe
ATP	Adenosine triphosphate
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
Da	Dalton
DO	Dissolved oxygen ($mgO_2\,L^{-1}$)
EBPR	Enhanced biological phosphorus removal
$FADH_2$	Flavin adenine dinucleotide
FDA	Food and Drug Administration
FF	Feast and famine
FID	Flame ionization detector
FSS	Fixed suspended solids ($mg\,L^{-1}$)
GAO	Glycogen accumulating organisms
GTP	Guanosine-5'-triphosphate
HA	R-hydroxyalkanoic acid
HB	3-hydroxybutyrate
HHx	3-hydroxyhexanoate
HRT	Hydraulic retention time (d)
HV	3-hydroxyvalerate
lcl-PHA	Long chain length PHA
mcl-PHA	Medium chain length PHA
MMCs	Mixed microbial cultures
N	Nitrogen (referred to nitrogen which was fed to the reactor)
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NPCM	Non-PHA cellular mass
OLR	Organic loading rate ($gCOD\,L^{-1}\,d^{-1}$)
OUR	Oxygen uptake rate ($mgO_2\,L^{-1}\,h^{-1}$)
PAO	Polyphosphate accumulating organisms
PGAP	PHA granule-associated protein
PHAs	Polyhydroxyalkanoates
PhaA	β-ketothiolase
PhaB	NADPH-oxireductase
PhaC	PHA synthase
PhaE	Subunit of PHA synthase

PhaP	Phasins
PHB	Poly(3-hydroxybutyrate)
PHBHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PP	Polypropylene
Prod	Productivity (gPHA L^{-1} d^{-1})
RNA	Ribonucleic acid
$\mathbf{r_{PHA}}$	Polyhydroxyalkanoates storage rate ($gCOD_{PHA}$ L^{-1} h^{-1})
$\mathbf{r_S}$	Substrate consumption rate ($gCOD_{VFA}$ L^{-1} h^{-1})
SBR	Sequencing batch reactor
scl-PHA	Short chain length PHA
SDS	Sodium dodecyl sulfate
SRT	Sludge retention time (d)
SS	Suspended solids
TCA	Tricarboxylic acid
TSS	Total suspended solids (mg L^{-1})
VFA	Volatile fatty acids
VSS	Volatile suspended solids (mg L^{-1})
$\mathbf{VSS_{non\text{-}polymer}}$	Volatile suspended solids without polymer (mgCOD L^{-1})
WWTP	Wastewater-treatment plant
$\mathbf{X_A}$	Active biomass (mgCOD L^{-1})
$\mathbf{Y_{OBS}}$	Observed yield ($gCOD_X$ $gCOD_{VFA}^{-1}$)
$\mathbf{Y_{STO}}$	Storage yield ($gCOD_{PHA}$ $gCOD_{VFA}^{-1}$)

1 INTRODUCTION

1.1 PHA: Structure, properties and applications

Over the past decades, most of the plastics used worldwide have been produced using unrenewable resources such as petroleum. This occurrence has been increasingly concerning some countries due to the impact of the carbon dioxide (CO_2) emissions to the atmosphere as a result of the combustion of this resources, the influence they can have on fauna and flora and, last but not least, the recalcitrance towards microbial degradation of these materials (Keshavarz & Roy 2010; Chen 2010). In fact, according to the Association of Plastics Manufacturers in Europe (APME), over 20 million tonnes of plastic wastes were produced in Western Europe in 2002 alone, of which only 38 % were recycled or used as an energy source (D. Dionisi et al. 2005). Also, the total consumption of plastics has been increasing, with about 150 million tonnes of plastics being consumed around the world yearly and the growth is expected to last until 2020 (Castilho et al. 2009). The massive dependence on the fossil resources will definitely lead to the depletion of the reserves in the long-term and to an increase of the cost of all materials produced with these sources. Therefore, an alternative to the production of polypropylene (PP) is in order.

In order to produce less recalcitrant plastics, the idea of producing biopolymers such as polysaccharides, polylactates and polyhydroxyalkanoates (PHA) has arisen. Biopolymers such as the ones mentioned are considered to be natural, biocompatible and biodegradable which means that they will hardly accumulate in the environment because they can be fully and easily biodegraded to water (H_2O) and CO_2 by bacteria (Rehm 2010; Jendrossek & Handrick 2002; Lee 1996). The last group of biopolymers stated is believed to be among the most promising biopolymers researched nowadays (Villano et al. 2014). Owing to the monomer variation in the structure of the polymer, PHA usually presents a wide range of structural, mechanical and thermal properties (Albuquerque et al. 2011). In the future, it is believed that a process capable of efficiently producing PHA will address 3 major world problems: the shortage of petroleum for plastic materials production, the CO_2 emissions to the atmosphere as a result of fossil resources combustion and a global protection of the environment (Chen 2010).

Although the observation of PHA granules as refractile bodies in bacterial cells goes back at least to Beijerinck 1888, it was only in 1927 when PHA was discovered and described by Lemoigne (Braunegg et al. 1998). Later, the synthesis of PHA by mixed microbial cultures (MMC) was observed in wastewater treatment plants designed for enhanced biological phosphorus removal (EBPR) (Serafim et al. 2008; Wallen & Rohwedder 1974). This polyester is biologically synthesized by bacteria as an energy storage polymer using R-hydroxyalkanoic acid (HA) monomers with a general chemical structure shown in Figure 1.1.

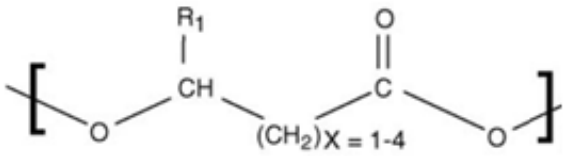

Figure 1.1 – PHA structure where X is the number of carbon atoms in the linear structure of the polymer and R the side chain. Adapted from (Keshavarz & Roy 2010).

Table 1.1 – Different types of homopolymers and their respective R-groups. These polymers are equivalent to the ones represented in Figure 1.1 with X = 1. Adapted from (Verlinden et al. 2007).

R-group	Full name	Abbreviature
CH_3	Poly(3-hydroxybutyrate)	PHB
C_2H_5	Poly(3-hydroxyvalerate)	PHV
C_3H_7	Poly(3-hydroxyhexanoate)	PHHx

The bacterial production of PHA is the only process that can fully guarantee a complete stereospecificity, due to the PHA synthase present in the cells, which is a requirement to ensure the typical biodegradability and biocompatibility of these polymers. This process, almost always, yields a polymer with R-configuration. A quite rare exception is only made for S monomers which were reported by *Sudesh et al* (Zinn & Hany 2005; Sudesh et al. 2000).

Up to this point, over 150 different monomers have been identified and described in the literature, which can be divided according to their composition and length (Zhang et al. 2006; Wang et al. 2014). These monomers may be divided into 3 classes: short-chain-length PHAs (scl-PHAs), which have a short side chain of only 3 to 5 carbons; medium-chain-length PHAs (mcl-PHAs) own a side chain of 6 to 14 carbons; large-chain-length PHAs (lcl-PHAs) that possess a side chain of 15 or more carbons (Zinn et al. 2001). Although there are three different categories of HA monomers and dozens of monomers, the last category is the least common and the least studied and only a few monomers have been widely studied (Kunasundari & Sudesh 2011). The most common and widely studied monomers so far are the shortest monomers, which are represented in Table 1.1.

Poly(3-hydroxybutyrate), PHB, is the most extensively studied polymer, so far, and it is a great example of a scl-PHA. This material is comparable to polypropylene in a few properties such as the melting temperature (177-179 °C), glass transition temperature, crystallinity and tensile strength as shown in Table 1.2. On the other hand, PHB possesses a very low extension to break when compared to polypropylene (Verlinden et al. 2007; Loo & Sudesh 2007). Regarding the degradation temperature, it is as high as 180 °C, which means that when this polymer is processed at temperatures as high as its melting point, there is a loss of the mechanical properties of the polymer due to substantial loss of molecular weight (Kunioka & Doi 1990).

Table 1.2 – Main properties of different PHA and PP. While PHBV contains 20 % of HV, PHB4B is made of 16 % of 4HB. Adapted from (Verlinden et al. 2007).

Parameter	PHB	PHBV	PHB4B	PP
Melting temperature (°C)	177	145	150	176
Glass transition temperature (°C)	2	-1	-7	-10
Crystallinity (%)	60	56	45	50-70
Tensile strength (MPa)	43	20	26	38
Extension to break (%)	5	50	444	400

Table 1.3 – Properties of PHBV copolymers with different HV molar composition (adapted from (Loo & Sudesh 2007)).

Properties	HV Composition (mol, %)					
	0	**3**	**9**	**14**	**20**	**25**
Melting temperature (°C)	179	170	162	150	145	137
Glass transition temperature (°C)	10	8	6	4	-1	-6
Young's Modulus (GPa)	3.5	2.9	1.9	1.5	1.2	0.7
Tensile strength (MPa)	40	38	37	35	32	30

Heteropolymers arise as a potential alternative to homopolymers such as PHB. These polymers consist of different monomers, and unlike PHB, they have improved properties when compared to PP, which can lead to improvements in some industrial applications (Verlinden et al. 2007). Usually, these heteropolymers are a mixture 3-hydroxybutyrate (HB) and other monomers, for instance, 3-hydroxyvalerate (HV) and 3-hydroxyhexanoate (HHx). The incorporation of monomers with a longer side chain such as the ones just reported has significant effect in the crystallinity, diminishing it (Reis et al. 2003). Additionally, these polymers have shown a higher flexibility, lower melting temperature and lower glass transition temperature. Although the previous properties have a dependence on the monomer composition, the temperature of degradation of the polymer does not seem to be significantly affected by the monomer composition (Bengtsson et al. 2010). Hence, the processing of the heteropolymer can be achieved without major molecular weight loss at lower temperatures when compared to processing of PHB (Carrasco et al. 2006).

Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is among the most studied copolymers, and as shown in Table 1.3, the properties of the polymer change along with the increase in the percentage of HV units. The shift in the properties of the polymer is a consequence of the polymer becoming more amorphous (Anderson & Dawes 1990). Poly(3-hydroxybutyrate-co-3hydroxyhexanoate) (PHBHx) shows a similar trend in properties' shift as the previous polymer, because of a loss of crystallinity (Doi et al. 1995). As previously reported, due to the high variability in structure and properties, tailor made polymer could have a deep impact on the world by suiting its wide range of needs (Linton et al. 2012). In fact, due to this polymer being produced by wide spectrum of over 300 bacteria ranging between Gram-positive and Gram-negative (e.g. *Lampropaedia, Pseudomonas, Rhodobacter, Ralstonia, Bacillus, Clostridium*), both aerobic and anaerobic photosynthetic bacteria and even some *Archea*, this biologically produced polymer may even show more variability and be tailor made by shifting some of the operational conditions (Valentino et al. 2011).

The field of applications of PHAs is considered to be extremely vast and believed to continue to expand over the years (Chen 2009). The early applications for the polymer were essentially its usage in packaging films, especially in bags, containers and paper coatings. Besides these common applications, there are other applications such as its use in disposable items such as diapers and shampoo bottles (Reddy et al. 2003). The use of PHA has already been extended to other fields such as medicine and pharmacy, where it has been studied as a drug carrier, a source for chiral compound synthesis or human implants, surgical sutures and blood vessel replacements (Chen 2009; Chen 2010; Tokiwa & Calabia 2004; Reddy et al. 2003). In fact, according to Chen, there is a polymer which has already received Food and Drug Administration's (FDA) approval as an implant biomaterial (Chen 2010). Other potential fields of PHA applications already reported in the literature are its use as a biofuel and the use of PHA monomers as drugs (Chen 2010). Despite the quite

large number of applications for PHA, and due to its selling price being still too high, a few other applications, either less expensive or high value applications, should emerge shortly, making them more widely used.

Despite the facts that PP is one of the most produced polymers nowadays and that it shares most of its mechanical and thermal properties with PHA, biodegradability is the one property that is exclusive to PHA. The molecular weight of the polymer plays an important role in PHA production because, not only does the molecular weight of the polymer matter in order to the polymer present the characteristics already mentioned, but it also affects for a matter of biodegradability. A vast number of microorganisms can degrade the polymer by using extracellular PHA depolymerases which partially degrade the polymer into small water soluble fragments, which after being incorporated, bacteria are able to completely degrade them internally (Madison & Huisman 1999; Khanna & Srivastava 2005). Although this process can be achieved without any special environmental requirements, both the activity of the enzymes is affected by pH, temperature, among other factors and the nature of polymer may also interfere with the rate at which it is degraded (Tokiwa & Calabia 2004; Madison & Huisman 1999). It has been reported that the lower the molecular weight and the melting point of the polymer, the higher the susceptibility for this polymer to be degraded in nature. Besides, the opposite trend has been described regarding crystallinity, which means that highly ordered structures have lower biodegradability (Philip et al. 2007).

1.2 Pure cultures

After the discovery and characterisation of PHA almost a century ago, this polymer has been thoroughly studied and an efficient and robust bioprocess for PHA production has also been researched in order to take advantage from all benefits this polymer presents when compared to PP. The maximum PHA productivity already achieved has been through the use of pure cultures. In the industry, PHA is generally produced in fed-batch mode in a two-step process: firstly, the reactor is fed with a sterile growth media in order to achieve a high cellular content; secondly, growth limiting conditions are deliberately imposed with a limitation of a nutrient (N, P or O_2) so that PHA storage is induced (Reis et al. 2011; Laycock et al. 2014). This process is accomplished by a few species with a considered high volumetric productivity such as *Cupriavidus Necator* (also known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Pseudomonas oleovorans*, *Protomonas extorquens* and *Alcaligenes latus.* Regarding the recombinant cultures, since these can grow and produce polymer at the same time, a nutrient limited phase is not required to induce PHA production. However, a nutrient feeding strategy must be developed in order to achieve a balance between cellular growth and polymer formation (Valentino et al. 2011). *E. coli* is the most used organism in genetic manipulation due to its convenience for genetic manipulation and fast growth. Besides, depending on the genes incorporated in the genome of the wild strain, high diversity in the structures of the produced PHA have been reported in the literature (Chen 2009). Maximum PHA contents up to 80-90 % of cell dry weight have already been reported in the literature as well as a substantially high volumetric productivity (up to 5 gPHB L^{-1} h^{-1}) (Reis et al. 2011; Reddy et al. 2003). Despite the yields stated above, the costs of the process, including the product extraction and isolation, are as high as €11.5-14 kg-PHB^{-1}, becoming an impediment to a widespread of products made of this polymer (Koller et al. 2011; Chanprateep 2010). The price is mainly due to substrate cost, accounting to approximately 40 % of the cost of the whole process (Albuquerque et al. 2007). The high cost attributed to the substrate is explained by the requirement of the sterilization of the nutrients, the use of refined sugar feedstocks such as glucose and sucrose and by the oxygen demand of this process. Moreover, the downstream-processing and energetic consumption contribute to the expensiveness reported (Dias et al. 2006; Reis et al. 2011).

Since the carbon source reasonably contributes to the costs of production as well as the extraction process, PHA production using genetically modified plants has come up. Not only the need for a carbon source is avoided because atmospheric carbon dioxide is used, but also plants are easier to harvest and less water is

required to be removed. However, PHA storage in recombinant plants has not been studied thoroughly enough and controversy over growth impairment as a result of PHA storage seems to occur in the literature (Poirier et al. 1995; Gumel et al. 2013). Another alternative which has already been studied, regards the use of photosynthetic MMC to produce PHA under a feast and famine regime (Fradinho, Oehmen, et al. 2013; Fradinho, Domingos, et al. 2013). Aeration is associated to reasonable portion of the costs of the process and a process which does not require aeration can be more economically competitive. Similar results to other studies using MMC under aeration and without photosynthetic organisms in PHA productivity have been reported. Although the aeration costs are cut with this process, high electricity costs can be associated because the organisms need constant light. Although sunlight as an illumination source has come up as potential cost-cutting measure, a long period of light absence occurs, decreasing the productivity of the process.

As a consequence of the overall high cost of PHA production, efforts have been made over the last years in order to turn the production into a more economical process, allowing the polymer to be used in its various applications (Laycock et al. 2014; Reis et al. 2011). In the field of genetic engineering, research has been done over the last years in order to obtain a higher PHA cellular content, more effective carbon source usage and to achieve innovative and valuable PHA properties (Vo et al. 2015). An increasing interest in using low-cost substrates based on agro-industrial wastes and by-products and MMC, which do not require sterile conditions, has also arisen (Valentino et al. 2011).

1.2.1 PHA synthesis

The metabolism of the most efficient PHA-accumulating organisms has been extensively studied and it is known that *C. necator* and *A. latus* use the Entner-Doudoroff pathway to convert carbohydrates into pyruvate in order to obtain energy in forms of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). Under unrestricted growth conditions, the pyruvate produced is metabolized into Acetyl coenzyme A (acetyl-CoA), which will enter the tricarboxylic acid cycle (TCA cycle). In this part of the catabolic pathway occurs the full oxidation of carbon into CO_2 coupled with the synthesis of guanosine-5'-triphosphate (GTP) and reducing agents, namely NADH and flavin adenine dinucleotide ($FADH_2$). Finally, in the mitochondria, NADH and $FADH_2$ are oxidised while oxygen is reduced to water in a process called oxidative phosphorylation. These two reactions result in an energy production which is used by the cell to produce ATP (Reis et al. 2011; Anderson & Dawes 1990).

On the other hand, when the cells undergo a lack of a nutrient such as N, growth is limited. Protein formation slows down, the concentration of RNA in the cytoplasm decrease and the cell is not able to continue to growth as fast as in the non-limiting nutrient media (Valentino et al. 2011). In fact, nitrogen limitation has as consequence the increase of the intracellular concentrations of NADH and nicotinamide adenine dinucleotide phosphate (NADPH), which lead to an inhibition of TCA cycle, and consequently, increasing the amount of acetyl-CoA in the cell (Reis et al. 2011). Hence when cells uptake more carbon than what they can use, cells metabolize it all to acetyl-CoA, then get rid of the excess by producing PHA. The described metabolic pathway for PHA formation is shown in Figure 1.2 as well as some other pathways used by different organisms. According to Figure 1.2, ultimately, the variety of polymers is almost limitless since a very wide range of substrates with unique chemical structures can be given to bacteria in order to produce different monomers, and consequently, polymers with high diversity.

Despite the structural differences of the proteins involved in the PHA production process, the steps of PHA production do not seem to differ much among different organisms. In *R. eutropha*, an extensively studied microorganism, PHB formation and degradation occurs according to the pathway represented in Figure 1.3. The carbon and energy stored can then be used according to the cell's needs by regulating both pathways. In

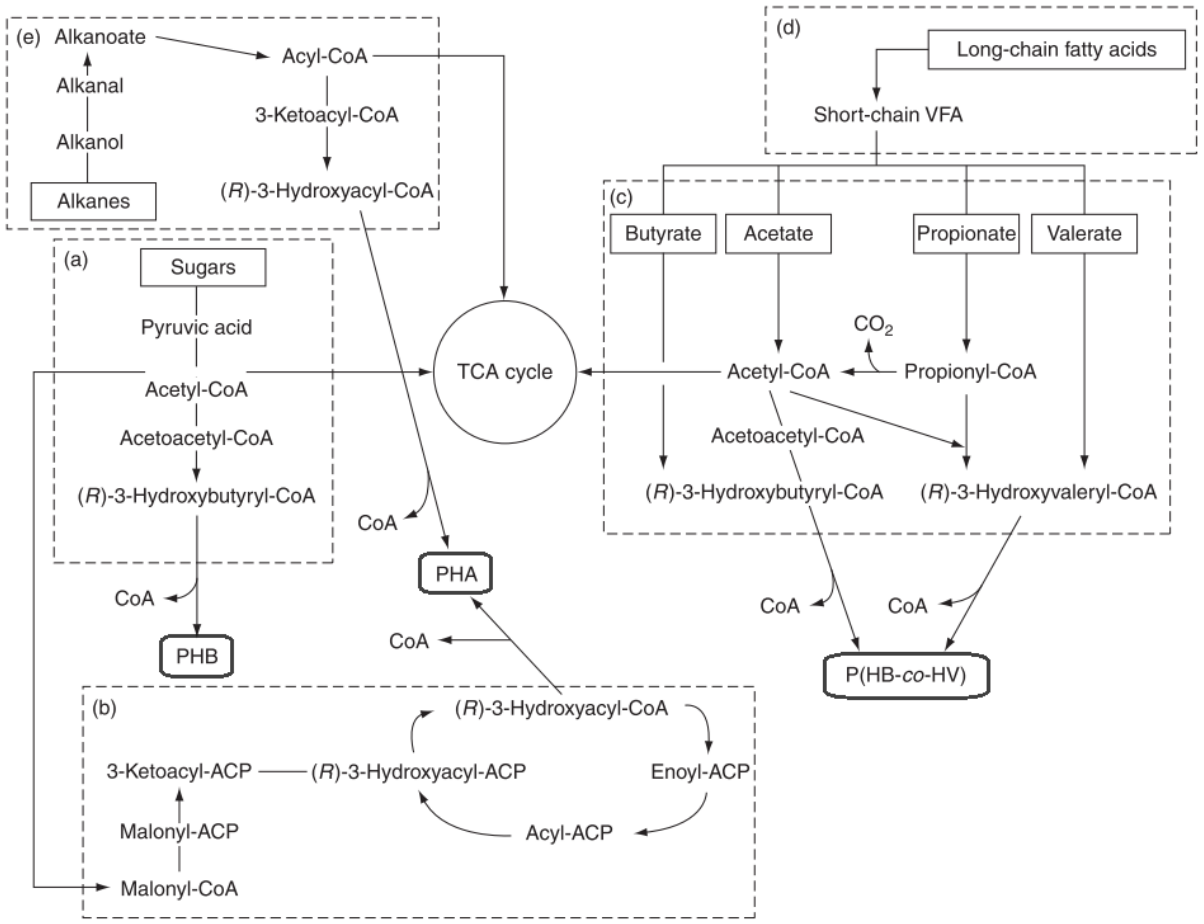

Figure 1.2 – Key metabolic pathways involved in PHA synthesis. As shown, the polymer can be stored through glycolysis (a), *de novo* fatty acid synthesis (b), β-oxidation of fatty acids (c, d) and alkanes' oxidation (e). Adapted from (Reis et al. 2011).

order to produce other monomers, instead of the pathway starting with acetyl-CoA specifically, the cell can initialize the pathway with other acyl coenzyme A (acyl-CoA) compounds which will lead to different monomers. Alternatively, in other microorganisms, different pathways can be used (Luengo et al. 2003). Although not all organisms can synthesize PHA using a large variety of monomers, some can due to possessing PHA synthases with different specificities or by having more than one PHA synthase. So far, three classes of PHA synthases have been described. The first type is a very well characterized PHA synthase, which is the one present in *R. eutropha*. Essentially, this polymerase is specific for short monomers, such as HA, 4-hydroxyalkanoate (4-HA) and 5-hydroxyalkanoate (5-HA). Despite the high specificity for short length monomers, it has been described that few medium length monomers (e.g. 3-HHx, 3-hydroxyoctanoate (3-HO) and 3-hydroxydodecanoate (3-HDD) can be added to the polymer with this PHA synthase. The two PHA synthases present in *P. oleovorans* are a good example of synthases which can add monomers with a range of 6 to 14 atoms of carbon. Lastly, the third type has been characterized based on the fact that this enzyme is made out of two functional subunits, designated PhaC and PhaE (Pötter & Steinbüchel 2005; Sudesh et al. 2000). The specificity of these enzymes is not well defined yet, but mostly seem to prefer the shorter monomers (Sudesh et al. 2000).

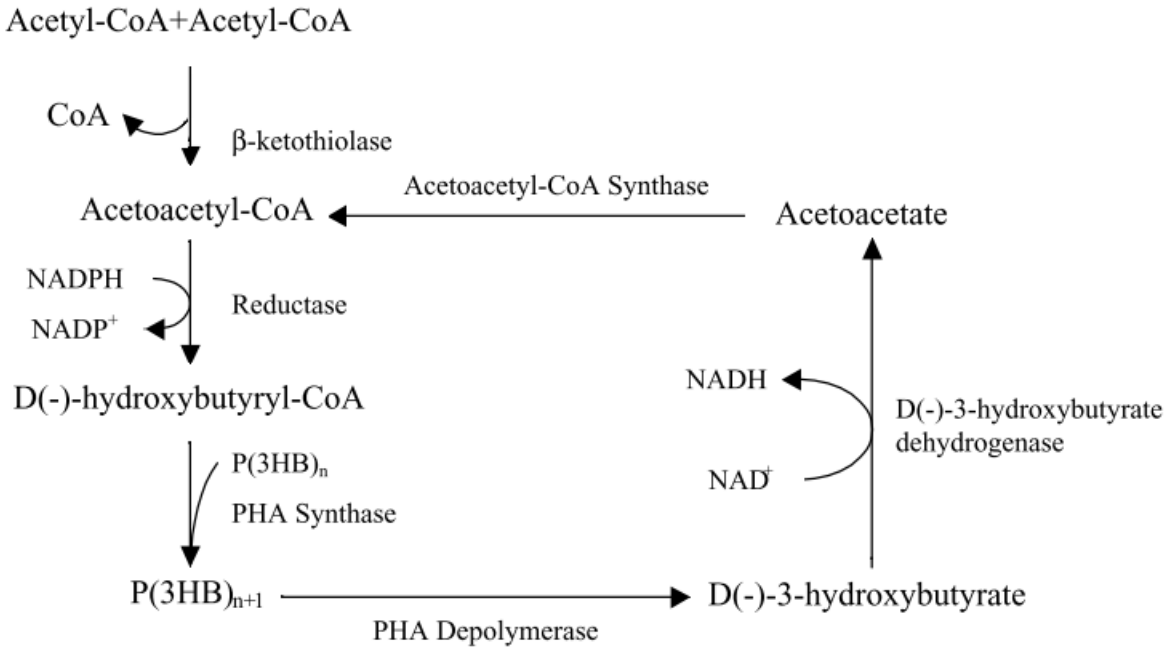

Figure 1.3 – Metabolic pathway which involves both synthesis and breakdown of P(HB). Adapted from (Khanna & Srivastava 2005).

The regulation of PHA production seems to be a complex process. In fact, it has been demonstrated that *B. megaterium*'s polymerase is synthesized in an inactive form and that it is required a polypeptide (PhaR) to active this enzyme suggesting the regulation may involve different environmental, metabolic and genetic signals (Luengo et al. 2003). The process in which PhaR either activates or deactivates polymer formation in *R. eutropha* has been described. This process also involves another PHA-binding protein, phasin (PhaP), which it has been demonstrated its important role in granule formation. This low-molecular weight protein enhances polymer formation by binding to granules and regulating their size, number and surface to ratio of PHB inclusions. Also, this modulation of size and number of granules seems to be controlled by the PHA synthase, PhaC (Luengo et al. 2003).Furthermore, phasins from various bacterial strains have been shown to increase PHA production, promote accumulation of PHA as numerous small granules, and when they are not present in the cell due to gene's deletion, this strains exhibit approximately 50 % decrease in PHA production relatively to the wild-type (York et al. 2001).

The three enzymes directly involved in the polymer synthesis belong to a single operon named phaCBA as shown in Figure 1.4. While phaA encodes for the β-ketothiolase (PhaA), phaB codifies the NADPH-oxireductase (PhaB) and phaC encodes the PHA synthase (PhaC) (Luengo et al. 2003). As previously pointed out, significant efforts have been made towards optimizing as much as possible the productivity of the process aiming at selling the product at a more reasonable price. Some work has been done on pure cultures while some other researchers have been working on genetically modifying organisms by inserting the crucial enzymes in the genome with the purpose of improving the process.

Another pathway that has been increasingly exploited is the pathway where short-chain organic acids – volatile fatty acids (VFAs) – are activated to acyl-CoA molecules and act as precursors for some HA monomers, as shown in Figure 1.2.

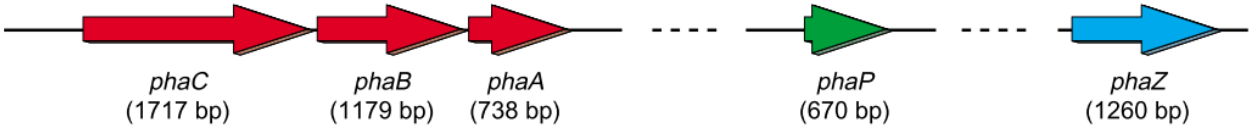

Figure 1.4 – Set of genes that codify the enzymes involved in PHA synthesis in *R. eutropha.* Adapted from (Luengo et al. 2003).

This pathway is common in MMC and, although there are few studies considering metabolic pathways in MMC using VFA, it has been assumed they share high similarity (Pardelha et al. 2014; Dias et al. 2008; Reis et al. 2003).

1.2.2 Granule Formation

PHA accumulate in the cell as water insoluble inclusions called PHA granules. Native PHA granules have been found to be made of 97.5 % PHA, 2 % proteins and probably some lipids. PHA granules seem to harbor a considerable amount of proteins called PHA granule-associated proteins (PGAP), among which there are PHA synthases, PHA depolymerases, regulators and structural proteins such as PhaP (Jendrossek & Pfeiffer 2014; Cai et al. 2014). Besides the already describe role for phasins, another phasin-like proteins have been described as crucial in the granule division during the cell division (Cai et al. 2014). This occurrence suggests granules are functional supramolecular complexes rather than a simple energy and carbon storage, for which the term “carbonosomes” was suggested to indicate the multifunctionality of the PHA granules (Jendrossek & Pfeiffer 2014; Jendrossek 2009). Although the number of granules seems to be constant over time and defined in the early stages of PHA accumulation, they present variable diameters (0.2 to 0.7 µm) (Valentino et al. 2011; Anderson & Dawes 1990; Braunegg et al. 1998). As stated by Anderson and Dawes, while the polymer can have a molecular weight up to 10^6 Dalton (Da), the PHA granules may weigh as much as $5*10^9$ Da, thus suggesting than each granule may have over 1000 molecules of polymer (Anderson & Dawes 1990). The PHA-storing bacteria, the feedstock and the growth conditions such as pH and temperature are among the factors that influence the final molecular weight of the polymer (Laycock et al. 2014; van der Walle et al. 2001; Kusaka et al. 1997). As the mechanical properties of PHA deteriorate when the molecular weight drops below $0.4*10^6$ Da, approaches to boost the average molecular weight have come up. While the first involves a genetic modification of the expression of PHA synthase, by taking advantage of the fact that low levels of this synthase lead to high molecular weight, the latter consists of poly(ethylene glycol) addition to the fermentation medium (Laycock et al. 2014; Sim et al. 1997).

1.3 Mixed Microbial Cultures as an economical alternative

The current industrial process for PHA production can hardly economically compete with the conventional petroleum-based plastics. Hence, MMC and cheaper carbon sources have been receiving more attention lately and they are being investigated with the intention to achieve a cost reduction (Pardelha et al. 2012). A combined use of MMC and waste carbon have the ability to stimulate a cost decrease due to a reduction in material and energy costs, both attributed to the lack of need of a sterilization of material and substrates (Jia et al. 2014). Moreover, they have a broader metabolic potential than single strains by feeding agro-industrial effluents to a mixed culture, and it even has the advantage of coupling PHA production with wastewater treatment (Valentino et al. 2011; Samorì et al. 2015). The use of economical carbon sources has been investigated over the past years, and countless research has been published since with diverse wastewaters effluents including fermentations of sugar cane molasses, cheese whey, pulp mill effluents, olive oil mill

wastewaters, paper industries and palm oil (Campanari et al. 2014; Bengtsson et al. 2010; Bengtsson, Werker, Welander, et al. 2008; Bengtsson, Werker, Christensson, et al. 2008; Salmiati et al. 2007). The availability of these substrates is quite variable though. While some wastes are obtainable the entire year, others are seasonal. Furthermore, the carbon concentration of these effluents is variable, ranging between a few grams of COD L^{-1} to hundreds.

The synthesis of PHA using MMC was first observed by Wallen *et al* in a wastewater treatment plant (WWTP) design for enhanced biological phosphorus removal in 1974 (Wallen & Rohwedder 1974). Typically, these systems work in alternated aerobic and anaerobic cycles and the bacteria selected from this conditions are glycogen-accumulating organisms (GAO) and phosphate accumulating organisms (PAO) (Serafim et al. 2008). Both organisms have similar metabolic pathways, being essentially distinguished by the ability of PAO accumulating poly-phosphate which GAO cannot. Since then, a lot of investigation has been performed in order to find out which organisms specifically are present in these processes and how it can be optimized.

The microbial population in MMC works in an open system, its composition is variable and it varies depending on the operational conditions imposed. It has been reported that nutrient concentration, pH, sludge retention time (SRT), among others affect both PHA production and microbial composition in this kind of systems. A proper selection either by discontinuous feed or a variation in the redox conditions (presence or absence of electron acceptor) can also have a great impact in microbial composition (Campanari et al. 2014; Valentino et al. 2014; Valentino et al. 2015; Villano, Beccari, et al. 2010; Lemos et al. 2008; Villano, Lampis, et al. 2010). Previous studies investigating microbial composition have revealed its wide diversity. A few *Alphaproteobacteria* (*Xantobacter* and *Curtobacterium*), several *Betaproteobacteria* (*Thauera*, *Alcaligenes*, *Comamonas*, *Achromobacter*) and *Gammaproteobacteria* (*Kluyvera*, *Pseudomonas*, *Acinetobacter*) were present in one study, while in another study *Paracoccus* of *Alphaproteobacteria*, *Thauera* and *Azoarcus* from *Betaproteobacteria* and *Flavobacterium* from *Flavobacteria* were present in higher concentration (Albuquerque et al. 2012; Lemos et al. 2008).

1.3.1 FF regime

In WWTP, selection processes were originally imposed for bulking control purposes and with the intention of avoiding problems related to biomass separation during sedimentation in EBPR systems. In addition to the usefulness of this strategy, later it has been found out that the key to the effectiveness of a PHA production process (both storage capacity and productivity) by MMC, relies on the culture selection (Valentino et al. 2011; Albuquerque et al. 2010). Whereas in PAO and GAO, selection was based on a metabolic shift, due to the presence or absence of oxygen, in other organisms, selection is accomplished through feast and famine (FF) conditions, which can also be called aerobic dynamic feeding (ADF), also implicating a metabolic change. In both scenarios growth limitation is accomplished while PHA production occurs. However, anaerobic and aerobic cycles result in a limitation because of the lack of O_2 whereas ADF results also in an internal limitation due to lack of substrate.

In a FF regime, there is a phase of abundance of carbon source and a following phase where it is absent (Reis et al. 2011). In the presence of carbon source, a fraction of substrate is aerobically oxidized to carbon dioxide, producing ATP and reduced compounds such as NADH, NADPH and $FADH_2$, which can be used for both growth and cell maintenance. Besides, an excess of carbon can be used for polymer storage, during this phase. As soon as carbon is depleted, the metabolism changes and bacteria must live with the carbon and energy stored during the previous phase. If the famine period is long enough, RNA and enzymes required for growth become produced in a smaller amount and, when carbon is available again, it may not ensure a growth rate as high as possible (Reis et al. 2011). This limitation is considered as internal because of the lack of internal compounds to grow. As growth deeply depends on protein production and this system is not working as much as it would with carbon fully available, when cells undergo a long depletion of carbon, a

growth response cannot be predominant after a few FF cycles (Van Loosdrecht & Heijnen 2002). In this case, a storage response takes over and, microorganisms, which can store polymer the fastest, will store more polymer during the feast phase and will be able to use it later for growth and maintenance, thereby having a greater competitive advantage over the other bacteria.

As a consequence of imposing a FF regime, microorganisms use the carbon source during the feast phase to grow and produce polymer and, consume it during famine phase for growth and maintenance (Van Loosdrecht et al. 1997). Figure 1.5 represents this situation by showing the period in which carbon is being consumed as well as polymer is being formed. As for growth, although there is a peak during feast phase, this process is far from negligible during the famine phase.

Although the feast and famine regime seems to select the PHA accumulating organisms, the efficiency of the method deeply depends on the FF ratio because if the famine phase is not long enough to ensure internal limitation, microorganisms will still be better fit to grow. As reviewed by Valentino *et al*, a FF ratio up to 0.26 led to a stable storage response, a FF ratio over 0.9 resulted in a growth response, whereas for intermediate values the culture was stable (Valentino et al. 2011). Although in order to achieve a higher productivity may require using a higher organic load rate (OLR), this factor may be detrimental to FF ratio, increasing it, as previously demonstrated in a couple of studies (Dionisi et al. 2006; Albuquerque et al. 2010). The SRT should also be considered, because both high SRT and low number of cycles per SRT will lead to lower FF ratio, thereby a better performance (Albuquerque et al. 2012; Johnson, van Loosdrecht, et al. 2010; Chang et al. 2012). Moreover, pH and temperature have been reported to directly impact the performance of the selection. While an increase in the pH from 7.5 to 9.5 resulted in a poorer storage rates and yields, a higher temperature (30 °C) seem to affect positively the feast phase when compared to the performance of the reactor at a lower temperature (15 °C) (Villano, Beccari, et al. 2010; Johnson, van Geest, et al. 2010; Valentino et al. 2011). Last but not least, nutrients may also alter the FF ratio, as nitrogen limitation can lead to an increase in the FF ratio, as Johnson *et al* demonstrated (Johnson, van Loosdrecht, et al. 2010).

1.3.2 MMC processes for PHA production

As previously explained, efforts have been made in order to discover economical alternatives processes for PHA production. The conversion of both wastewaters and synthetic VFA mixtures, by MMC, has been reported as a more inexpensive procedure to synthesize the polymer in comparison to pure cultures processes and poly-ethylene production (Gurieff & Lant 2007; Ince et al. 2012). Depending on the substrate employed, typically, this process is either performed in two or three independent steps (Albuquerque et al. 2007).

The two-step process contemplates an initial phase for culture selection in PHA-accumulating organisms and further step for polymer accumulation by the selected culture and it has been widely and successfully applied (Moralejo-Gárate et al. 2013; Johnson et al. 2009). The produced PHA is extracted and purified afterwards. This process is appropriate for PHA production when VFA's are used as feedstock. When glucose or wastewaters are used as feedstock, a preliminary stage is performed in order to convert the organic matter into appropriate substrates, such as VFA's (Valentino et al. 2011). This process occurs under anaerobic conditions and soluble organic matter is fermented into organic acids and other fermentation products such as alcohols, hydrogen and carbon dioxide (Serafim et al. 2008; Bouallagui et al. 2005). Tight regulation of the fermentation process must take place so that biogas formation is inhibited and most of organic matter is converted in the desired products. Afterwards, the obtained substrate can be used as feedstock in the subsequent two steps, both MMC selection and PHA accumulation, turning the two-step process into a three-step process (Duque et al. 2014). This requirement arises as a consequence of the inability of MMC to store PHA using sugar-rich feedstocks such as wastewaters (Dionisi et al. 2005).

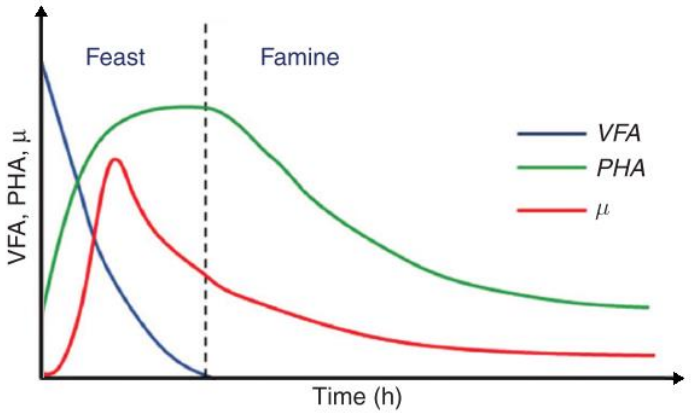

Figure 1.5 – Polymer storage under aerobic conditions in a feast and famine regime by MMC. Reprinted from (Reis et al. 2011)

The efficiency of the microbial selection stage is crucial for the overall production of polymer in the following stage. The aim of this phase is to select a culture able to produce a high amount of PHA in stable mode. Besides, a high growth rate is important so that volumetric productivity of the process is as high as possible (Reis et al. 2011). Over the years, several methods have been tried in order to achieve a proper culture selection. Selection is usually performed in SBR and the variable most studied in these methods is the aeration and the impact of absence of oxygen during the beginning of the cycle. The anaerobic/aerobic selection process consists of an initial absence of oxygen followed by full aeration of the reactor. Although PHA content between 30 and 57 % was obtained under anaerobic/aerobic conditions, this process was not stable (Takabatake et al. 2000). Satoh *et al* proposed a new method, in which aeration was always present throughout the whole time (Satoh et al. 1998). However, less oxygen was present in the beginning of the cycle, in order to hinder growth of microorganisms and promote PHA production in a microaerophilic environment. The produced PHA was consumed during a fully aerobic phase for growth and maintenance, yielding a maximum PHA content of 62 %. Once again, PHA production was not stable (Dias et al. 2006). PHA production by activated sludge under fully aerobic conditions can be achieved when the sludge goes through successive periods of feast and famine. It has been shown this method can result in a stable storage capacity (Serafim et al. 2004; L. S. Serafim et al. 2008). Although FF regime can also be obtained with plug flow reactors and in two continuous stirred-tank reactors (CSTR) in series with biomass recirculation, SBR is the most widely used (Valentino et al. 2011). The conditions that improve the selection stage and feast and famine ratio have been investigated over the last years and the most common parameters used are summed up in Table 1.4. This process may economically compete with the production of PHA from pure cultures with the advantages of being a simpler process and requiring less investment and operational costs. However, the efficiency of the selection stage using consecutive cycles of feast and famine directly depends on feast and famine ratio.

The last stage of the PHA production has the aim of maximize the intracellular amount of polymer, resulting in a high PHA content in the biomass. This process is often performed in fed-batch mode, using a high concentration of a carbon source and quite low in nutrients, causing a very high COD:N ratio. The availability of nutrients such as nitrogen can negatively impact the final PHA content due to resulting in a growth response and loss of storage response (Reis et al. 2011). So far, the maximum PHA content in the biomass (89 %) was obtained by Johnson *et al* and it is within the range previously obtained for pure cultures (80-90 %) (Reis et al. 2011; Johnson, Kleerebezem, et al. 2010).

Table 1.4 – Summary of some of the operating parameters typically used for MMC selection in PHA-storing organisms. Adapted from (Reis et al. 2011)

Operating Parameter	Range
Sludge retention time (d)	1-20
Hydraulic retention time (d)	1-3
Length of SBR cycle (h)	2-12
pH	7-9.5
T	20-30
Operation mode	SBR or two continuous reactors
Organic loading rate (gCOD L^{-1} d^{-1})	1.8-31.25
Substrate concentration (gCOD L^{-1})	0.9-31.25
COD:N ratio (gC gN^{-1})	9-120
F/F ratio	0.1-1.15

1.4 The importance of nutrients

Microorganisms must be provided with the full spectrum of both micronutrients and macronutrients in order to perform all kinds of cellular activity. Some nutrients have more impact on growth and polymer production though. For instance, N, P and S, have already been linked tightly to growth and their limitation has been directly correlated with growth limitation and they are known to affect PHA storage (Hong et al. 2000; Basak et al. 2011; Wang et al. 2007). Low Acetyl-CoA levels as well as reduced amount of RNA's and enzymes are a consequence of nutrient limitation and they have been reported to enhance PHA production and to affect the final PHA content (Zinn et al. 2001; Ince et al. 2012).

FF regime results in a successful PHA storage by causing internal growth limitation to the microorganisms. Selection of microorganisms with high storage capacity has also been demonstrated under FF regime with both nutrient excess and nutrient-limiting conditions (Serafim et al. 2004; Johnson et al. 2009). As reported in the literature, the growth yield in the famine phase as well as the ratio of carbon driven towards growth and PHA storage are affected by the COD:N ratio (Valentino et al. 2011). Several studies have reported that nutrient limitation can have direct influence on several parameters. Johnson *et al* found that higher PHA contents can be obtained under nitrogen limitation, while carbon limitation favours enrichment and long-term cultivation of the microorganisms (Johnson, van Loosdrecht, et al. 2010) Hong *et al* and Wen and colleagues have demonstrated that a shift in the COD:N ratio can be enough to affect the monomer composition of the polymer (Hong et al. 2000; Wen et al. 2010). Furthermore, a few studies have reported that, nutrient limitation, as a result of phosphorus and nitrogen limitation, can be the best strategy for PHA production (Johnson, Kleerebezem, et al. 2010; Valentino et al. 2015; Venkateswar Reddy & Venkata Mohan 2012). However, an excess nutrient has been associated with a higher biomass yield, thereby increasing the biomass volumetric productivity (Reis et al. 2011).

As a result of PHA production by MMC being ultimately aiming at using wastewaters as a substrate and these wastewaters having varying nutrient concentrations, the study of the impact of nutrients on the process performance becomes important so that SBR and accumulation reactors are not reasonably affected by the feedstock composition (Johnson, Kleerebezem, et al. 2010; Valentino et al. 2015).

1.5 Polymer Extraction

Ultimately, the produced polymer must be extracted and purified in order to be used later. The costs of this part of the process are considerable and different processes have been studied over the years in order to maximize purity, minimize costs and obtain a sustainable method (Reis et al. 2011).

The use of solvents is one of the oldest and the most widely used method to extract PHA from microorganisms. It is a recurrent method in laboratories due to its rapidity and simplicity and it can be divided into two steps. Firstly, the permeability of the cellular membrane is modified, thereby allowing the contents to be released and solubilized. Lastly, a non-solvent precipitation is followed (Jacquel et al. 2008; Kunasundari & Sudesh 2011). The process can be done using chlorinated hydrocarbons, for instance, chloroform and 1,2-dichloroethane and cyclic carbonates such as ethylene carbonate 1,2-propylene carbonate (Kunasundari & Sudesh 2011). Using this method, it is possible to obtain a polymer with high purity, high molecular weight with low degradation. On the other hand, the recovery yield is not high enough and the costs and the environmental impact do not allow the method to be unanimously accepted. Therefore, other approaches such as digestion methods, among others, have been considered as potential alternatives (Jacquel et al. 2008; Kunasundari & Sudesh 2011).

Digestion methods involve the solubilisation of non-PHA cellular mass (NPCM) and they can be classified as chemical digestion and enzymatic digestion, depending on the agent employed. Regarding chemical digestion, sodium hypochlorite and surfactants such as sodium dodecyl sulfate (SDS) and Triton X-100 are typically used. The first agent owns strong oxidizing properties and non-selectivity, allowing PHA recovery by digestion of the NPCM, while the second promotes disruption of the cellular wall by incorporating itself in it. While sodium hypochlorite allows an extraction with high purity, it can cause polymer degradation by as much as 50 % of polymer mass. On the other hand, surfactants do not typically cause loss of the molecular weight, but the final purity is low (Kunasundari & Sudesh 2011; Jacquel et al. 2008). As reviewed by Kunasundari and Sudesh, more complex strategies such as a sequence of surfactant and sodium hypochlorite and the use of chloroform with sodium hypochlorite have been tried (Kunasundari & Sudesh 2011). They resulted in a better, rapid and more economic recovery of PHA when compared to solvent extraction and a way to retain the high molecular weight of the polymer, respectively. Enzymatic digestion is based on the use of hydrolytic enzymes which specifically destroy the cellular membrane. The method gives rise to high purity and good polymer recovery with high reaction rates and negligent damage to the polymer. However, the high costs and complexity of the recovery process have been crucial drawbacks, thereby hampering its diffusion as a widely used method for PHA recovery (Kapritchkoff et al. 2006; Gumel et al. 2013; Kunasundari & Sudesh 2011). As reviewed by Jacquel and colleagues, other methods have also been studied such as mechanical disruption and supercritical fluids, but they are not widely used or they are still emerging and underdeveloped technologies requiring further research and optimization (Jacquel et al. 2008).

The cost of the extraction is strongly correlated with the PHA content, in other words, the more average PHA cell content in the biomass, the less expensive and more efficient the extraction will be. This is a consequence of the requirement of less digesting agent (Lee & Choi 1998; Reis et al. 2011). As a result of a fairly low volumetric productivity and PHA content of MMC systems when compared to pure cultures, the optimization of the extraction process is even more important and it may be proven critical for economic viability of this process.

2 OBJECTIVES

As previously stated, most plastics used nowadays are made using non-renewable sources. Although they are economic, they are not biodegradable, thereby accumulating in the environment and directly affecting the ecosystem. PHA is one of the most promising alternatives to change the current situation, and this work ultimately aims at finding an eco-friendly and economic process for PHA production. Since the current processes for PHA production are still underdeveloped causing the prices to augment, performance should be optimised by increasing the volumetric PHA production rate, and/or decreasing costs in order to be economically competitive.

Whenever MMC are used, low-cost renewable feedstocks can also be used and these are often nutrient deficient (such as paper mill and olive oil mill wastewaters, cheese whey permeate, molasses), requiring nutrient supply in the selection stage in order to allow microbial growth. The effect of nutrient limitation on the performance of both the SBR and the PHA accumulation reactor has been widely studied, with main reference to COD:N ratio.

Despite the fact that it is already known a range of concentrations of nitrogen which yield a good performance, as well as COD:N ratios which induce a good selection stage, there is still lack of knowledge on the impact of nitrogen supply strategies in the SBR on the process performance (Johnson, van Loosdrecht, et al. 2010).

In this context, the main aims of this study were to investigate:

- the possibility to uncouple nitrogen supply from carbon feed within the SBR cycle, in order to make nitrogen available after the feast phase. As a result of this change, theoretically, microorganisms would only have nitrogen during the famine phase, thereby growing only during this period. In contrast, during feast phase, microorganisms would use all the external carbon to produce polymer, and use it to survive during the following phase;
- the effect of different COD:N ratios (100:2.43, 100:3.03 and 100:3.79) in a SBR operated with uncoupled carbon feed and nitrogen supply.

In order to accomplish these objectives, a lab-scale SBR has been completely assembled. The reactor was monitored through a software which allowed to control each phase of the SBR cycle. A synthetic mixture of VFA, consisting of acetic and propionic acid, was used as carbon feedstock at an overall OLR of 8.5 gCOD L^{-1} d^{-1}. Biomass selected in the SBR has been also used to perform PHA accumulation tests.

The selection stage would be considered a success in case of achieving a selection of microorganisms able to grow fast (great growth response), as well as produce PHA quickly and in a large amount (strong storage response). The SBR performance was assessed both in terms of biomass and PHA production and the obtained results were compared to those of a previous study done by Valentino and colleagues, which was carried out with coupled feeding of carbon and nitrogen sources (Valentino et al. 2014).

3 MATERIALS AND METHODS

3.1 Experimental set-up

3.1.1 MMC selection in Sequential Batch Reactor (SBR)

The culture selection stage was conducted in a lab-scale SBR with a working volume of 1 L. The reactor was inoculated with activated sludge from the Roma Nord full-scale treatment plant which mostly treats domestic wastewater. The reactor was operated under aerobic conditions at controlled temperature (25 °C) and pH (7.6) and a schematic representation of the SBR is shown in Figure 3.1. The reactor was made of glass with the top being made of plexiglass, it had 8 apertures, which were used to add and withdraw the mixed liquor, aerate the reactor and allow probes to access the reactor for monitoring purposes, such as the oxygen and pH probes. The reactor was aerated by means of air pumps through three ceramic diffusors and stirred by a mechanical impeller. As a result, it was guaranteed a non-oxygen limiting concentration (> 2 mg L^{-1}) and an oxygen transference coefficient (K_La) of about 0.3 min^{-1}. While the pH of the reactor was kept constant through carbon dioxide bubbling through compressed gas cylinder, temperature remained at around 25 °C using a thermostatic jacket.

The reactor was controlled with a computer software which allowed the management of all the phases whose total length was maintained at 6 h throughout all the study. The SBR was operated with nitrogen supply uncoupled from carbon feed within each cycle and three runs were performed at different COD:N ratios (gCOD:gN): 100:3.79, 100:3.03 and 100:2.43. The structure of the cycle was the same for the first two runs and consisted of a carbon feed phase, (10 min; 0.21 L $cycle^{-1}$), a reaction phase when the carbon was consumed (140 min), a withdrawal phase of the mixed liquor (2 min; 0.25 L $cycle^{-1}$), a nitrogen feed phase (5 min; 0.04 L $cycle^{-1}$) and a famine phase (203 min). On the other hand, the third run started with the same exact conditions but on the 8th day of operation the withdrawal phase was moved to the end of cycle. Thus, the cycle structure started with the carbon feed (10 min; 0.21 L $cycle^{-1}$), followed by a reaction phase (142 min), nitrogen feed (5 min; 0.04 L $cycle^{-1}$), famine phase (201 min) and, lastly, withdrawal phase (2 min, 0.25 L $cycle^{-1}$). This change occurred because the carbon was not depleted by the moment withdrawal occurred, resulting in the withdrawal of carbon, so this phase as well as nitrogen feed were postponed, extending the reaction phase. Unlike common SBR operations, settling of the biomass was not implemented. Therefore, SRT and hydraulic retention time (HRT) were both equal to 1 d.

During the reaction phase, carbon is present abundantly until it is depleted and PHA is produced at the carbon source expense. On the opposite, the famine phase occurs when external carbon is depleted and biomass grows using the nitrogen that was just fed and the carbon in the form of polymer that was previously accumulated. The volume withdrawn during the withdrawal phase is equal to the sum of the volumes added during the carbon and the nitrogen feeds. The reactor was under stirring and aerations during all times except for the withdrawal phase in which aeration was stopped to guarantee the right volume was withdrawn.

Regarding the carbon source, a synthetic mixture of acetic (85 % on a COD basis) and propionic acids (15 %) was fed in all runs, with an organic load rate (OLR) equal to 8.5 gCOD L^{-1} d^{-1} (corresponding to 260 Cmmol L^{-1} d^{-1}). While the carbon source was constant during all times for all runs, the nitrogen load varied among runs. For the three separate runs, the addition of nitrogen was performed using ammonium sulfate and, in the first run, 322 mgN L^{-1} d^{-1} (corresponding to 23.0 Nmmol L^{-1} d^{-1}) were given to the reactor, in the second, only 258 mgN L^{-1} d^{-1} (or 18.6 Nmmol L^{-1} d^{-1}), and in the latter, 206 mgN L^{-1} d^{-1} (equal to 15.0 Nmmol L^{-1} d^{-1}). As a result, the COD:N ratio (gCOD:gN) in the runs was 100:3.79, 100:3.03 and 100:2.43, respectively, which, on a molar basis, corresponded to 11.3, 14.0, and 17.3 Cmol $Nmol^{-1}$). Along with the carbon source, the mineral medium was administrated to the biomass. Its composition was as follows (mg L^{-}

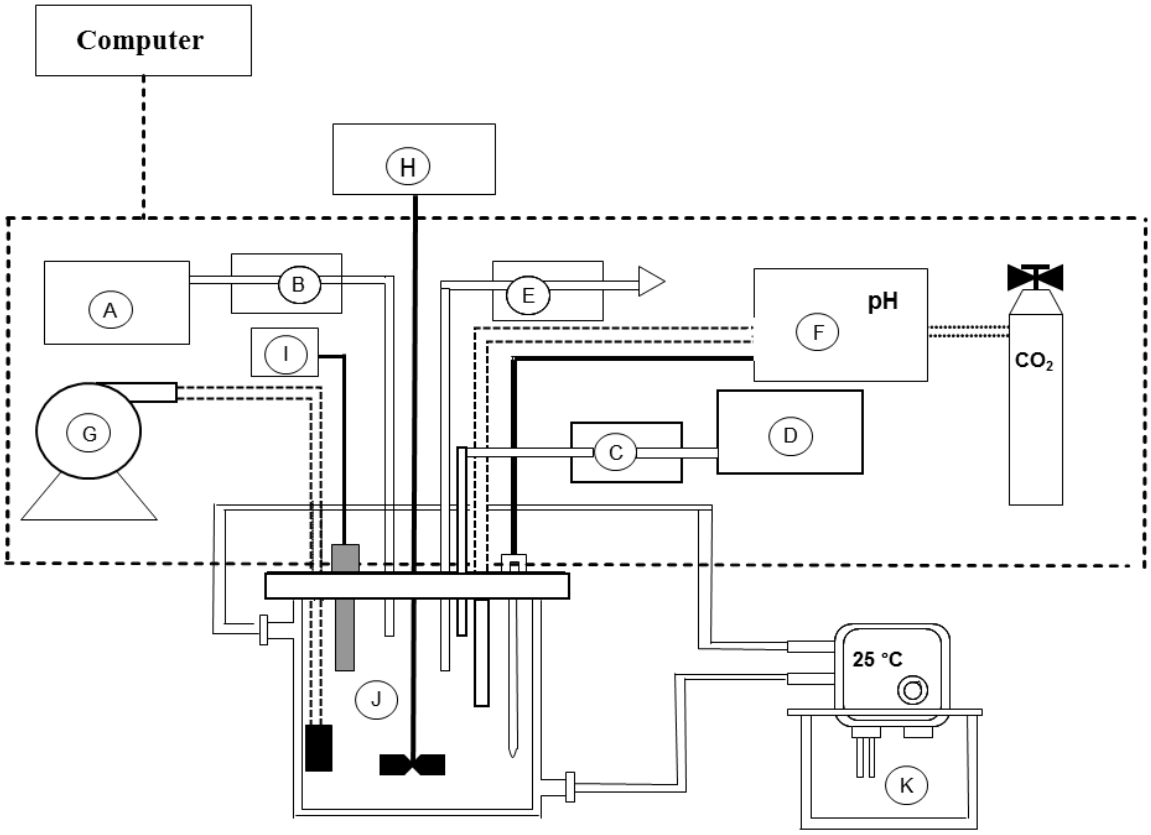

Figure 3.1 – Layout of the SBR reactor: A – Feed tank; B – peristaltic pump for substrate feeding; C – peristaltic pump for nitrogen feeding; D – nitrogen feed tank; E – withdrawal pump; F – CO_2 pump and pH meter; G – air pump; H – mechanical stirrer; I – dissolved oxygen meter; J – reactor vessel; K – thermostatic vessel.

[1]): $FeCl_3 \cdot 6H_2O$ (2), EDTA (3), thiourea (20), K_2HPO_4 (335), KH_2PO_4 (259), $MgSO_4 \cdot 7H_2O$ (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), $NiCl_2 \cdot 6H_2O$ (0.02) and $Na_2MoO_4 \cdot 6H_2O$ (0.03).

The SBR performance was monitored through daily sampling and measurements of total suspended solids (TSS), volatile suspended solids (VSS), polymer concentration and composition and nitrogen concentration. Every day, PHA and nitrogen concentrations were measured five times, corresponding to the beginning of the cycle, the end of feed, end of the feast phase, end of the nitrogen feed and end of cycle, whereas suspended solids (SS) were measured three times per day, which corresponded to the beginning of the cycle, end of the feast phase and end of the cycle. With the purpose of obtaining detailed profiles of the variations of PHA and substrates over time, kinetic tests were conducted. In these tests, daily sampling remained unchanged and one sample for polymer and nitrogen every half hour was added within the SBR cycle. Additionally, sample for organic acids was also taken every time sample for nitrogen was taken. Kinetic tests were done just once during the first run, three times during the second one and twice in the last run. For run, it is meant as the period of operation when each of the COD:N ratio was applied. First run corresponds to the highest nitrogen load (N-FAMINE 1), whereas the third run corresponds to the lowest (N-FAMINE 3). Second run is a middle ground.

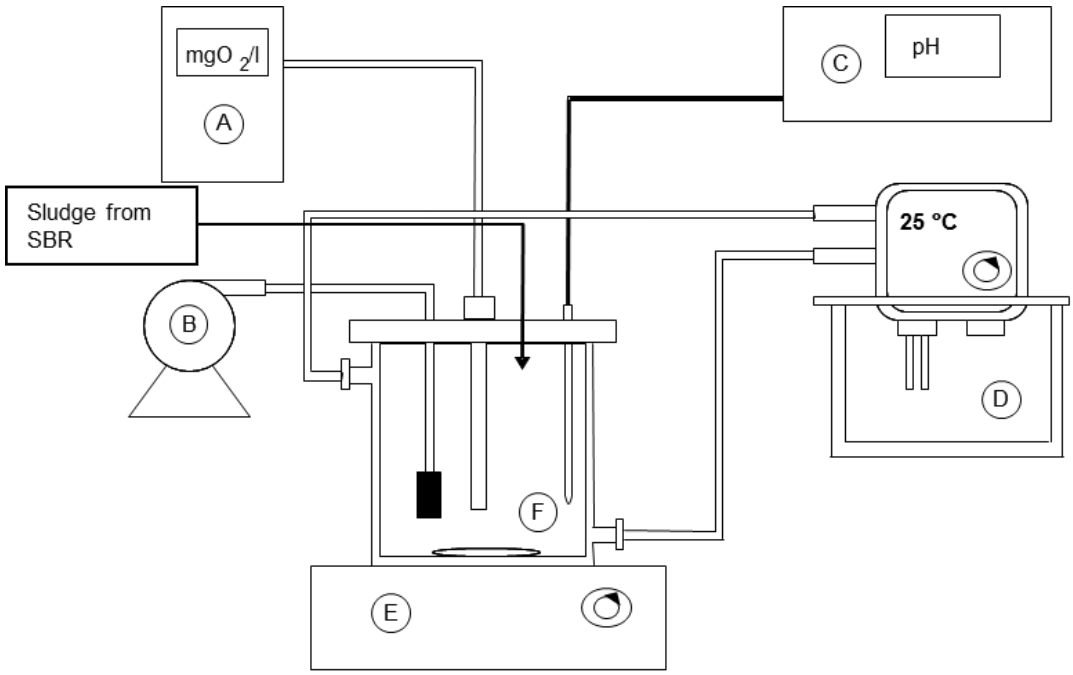

Figure 3.2 – Layout of the accumulation reactor: A – dissolved oxygen meter; B – air pump; C – pH meter; D – thermostatic bath; E - magnetic stirrer; F – reactor vessel

3.1.2 Polymer accumulation in batch reactor

With the intention of simulating the last stage of the process, the PHA accumulation stage was performed in a 0.5 L batch reactor at 25 °C, which was achieved using also a thermostatic jacket. In general, the maintenance of the batch reactor was quiet similar to the maintenance of the SBR, which means that the reactor was aerated using an air pump, pH and oxygen probes were inserted in the reactor for control purposes and mechanical stirring was implemented. However, pH was manually adjusted at 7.6 by means of concentrated solutions of sodium hydroxide (3 M) and sulfuric acid (0.8 M). A schematic representation of the accumulation reactor is shown in Figure 3.2**.**

Typically, by the moment the carbon was depleted in the SBR, 125 mL of sludge were withdrawn from the SBR to the batch reactor and the accumulation stage started from this point on, having a total duration of 6 hours. The sludge was then diluted with 375 mL of mineral medium (having the same composition of that used in the SBR), adding up to the total volume of the reactor. During each accumulation test, a very concentrated (500 gCOD L^{-1}) solution of synthetic acids, with the same proportions of acetic and propionic acids as in the SBR, was made and used for the feeding. More specifically, 1 mL was spiked at the beginning of the test and 0.5 mL were also fed whenever carbon was depleted, which was determined by an decrease in the oxygen uptake rate (OUR). It resulted in an initial concentration of acids equal to 1 gCOD L^{-1} and a second addition of 0.5 gCOD L^{-1}. Only once, this additional spike was not given. No nitrogen was fed in the accumulation reactor, therefore no nitrogen was present during the whole period of polymer accumulation.

The performance of the accumulation batch reactor was evaluated by means of sampling for SS, PHA and substrate at the beginning of each test, both before and after substrate feeding, and at regular intervals of 20 minutes along the test, except for SS, which was only sampled at the beginning and at the end of each batch test. Batch accumulation reactor was set up as soon as the SBR performance was considered stable. Due to

some delay with sample analyzing in the first run, consequently lack of knowledge about the SBR's stability, along with some punctual instability, no batch was performed in the first run. In the second run, 3 batch tests were performed, whereas in the third run, no batch test was performed thanks to a poor SBR performance which will be later described in detail.

3.2 Analytical procedures

3.2.1 Suspended solids determination

Measurement of TSS was consistent with standard methods (APHA, 1995), except for the volume which was sampled. Due to technical reasons, only 5 mL were sampled. This volume, which was sampled, was filtered under vacuum through GF/C filters (47 mm of diameter and 10 µm of porosity). Before filtration, filters were pretreated at 100 °C in incubator for 24 h. Their weight (P_1) was measured and, after sample filtration, they were incubated for at least 4 h at 100 °C and then for 30 min in exsicator and weighted (P_2). Lastly, filters were put in a muffle at 550 °C for 30 min, kept in the exsicator for 30 min and weighted (P_3) one last time. TSS, fixed suspended solids (FSS) and VSS were calculated, in mg L^{-1}, as follows:

$$TSS = \frac{(P_2 - P_1)}{V} \qquad FSS = \frac{(P_3 - P_1)}{V} \qquad VSS = TSS - FSS$$

Regarding non-polymer VSS ($VSS_{non\text{-}polymer}$), which has also been called in literature as active biomass (X_A), this parameter is obtained by the subtraction of VSS by the polymer (both measured at the same moment within the SBR cycle, i.e. at the end of feast phase). In order to convert it to gCOD L^{-1}, the oxidation stoichiometry coefficient of the active biomass is used (1.42 gCOD $g_{X_A}^{-1}$).

3.2.2 PHA determination

With the aim of measuring the PHA concentration, 5 mL of mixed liquor were taken at different moments of the SBR cycle and they were immediately treated with 1 mL of NaClO (7 % of active Cl_2). The samples were then stored at -4 °C until the day they were analyzed. The protocol used for extraction, hydrolysis and esterification of the HA was similar to the one used by Braunegg and colleagues (Braunegg et al. 1978). Unfrozen samples were centrifuged at 10000 rpm for 30 min with the aim to separate polymer from most of the cellular contents. 2 mL of a solution containing methanol, benzoic acid (50 mg L^{-1}) and acidified with sulfuric acid 3 % (v/v) were added to the pellet recovered from the previous procedure. 1 mL of chloroform was added to the samples and, the mixture was put in a thermostat for 4 h at 100 °C. Finally, after cooling at room temperature, 1 mL of water was added and samples were mixed for 10 minutes. As a consequence, the mixture separated into a double phase, where the aqueous phase contained some cellular content and the organic phase, the esters which resulted from the esterification reaction.

A gas chromatograph (Perkin Elmer 8410 Gas Chromatograph) equipped with a flame ionization detector (FID) set to 200 °C, a 6 ft · ¼ inch · 2 mm glass column with 2 % Reoplex 400 on 60/80 Chromosorb GAW was used for polymer quantification. Temperature started at 100 °C and rose at 8 °C min^{-1} until 148 °C. N_2 was used as carrier gas at 120 KPa, whereas H_2 and air pressure remained at 120 °C and 150 °C, respectively. The injector temperature was set to 200 °C. Data was processed by means of a computing integrator (SEPU 3010 Chromatography Station). The retention times of the monomers (HB and HV) and internal standard (benzoic acid) corresponded to 4.5 min, 5.6 min and 6.5 min, respectively. The analysis consisted of an injection of 1 µL of the organic phase in the gas-chromatograph.

Calibration standards were made using a copolymer of P(HB-HV) containing 5 % (w/w) of HV content (Sigma) with concentrations ranging between 0 and 3000 mg L^{-1}. Their preparation was done exactly the

same as the samples, with the only exception made for the centrifugation. Due to benzoic acid being used as internal standard, the determined standard curves reported the concentration of each monomer as a function of the ratio of peak area of the corresponding monomer and that of internal standard.

The results were obtained in mg L^{-1} and they were then converted into mgCOD L^{-1}, according to their coefficients: 1.67 mgCOD $mgHB^{-1}$ and 1.92 mgCOD $mgHV^{-1}$. While the PHA concentration was the sum of the concentrations of both monomers, the PHA content in the biomass was calculated by dividing the PHA concentration by the VSS concentration and it was expressed as a percentage.

3.2.3 VFA Analysis

Sampling for VFA analysis was achieved by taking 2 mL of sludge from the reactor with a syringe, filtrating them through a filter of cellulose acetate with a porosity of 0.45 µm. Samples were then stored at 4 °C until the moment of the analysis. Regarding the preparation of the analysis, 100 µL of oxalic acid (0.33 M) and 100 µL of acrylic acid (2 g L^{-1}; internal standard) were added to 1 mL of sample for the analysis. Whenever necessary, samples were diluted.

Analysis were performed in a gas chromatograph (DANI Master GC) equipped with a FID set to 200 °C and a 200 cm glass column with 2 mm in diameter. A packed stationary phase Carbopack was used and the temperature was set at 175 °C. Helium was used as a carrier gas at a flow rate of 25 mL min^{-1}. The computer software Clarity was used to integrate the chromatograms and the typical retention times were 3 min, 7 min and 9 min for the acetic, propionic and acrylic acid, respectively.

Calibration curves were done after a preparation of standards with both acids with concentrations ranging from 0 to 210 mg L^{-1} and from 0 to 100 mg L^{-1} for acetic and propionic acid, respectively. Due to acrylic acid being used as internal standard, calibration curves reported the concentration of each VFA as a function of the ratio between the peak area of the corresponding acid and that of acrylic acid. The results were obtained in mg L^{-1} and then converted into mgCOD L^{-1} according to their conversion coefficients: 1.067 mgCOD mg_{acetic}^{-1} and 1.51 mgCOD $mg_{propionic}^{-1}$. All coefficients have been calculated based on the equations of full carbon oxidation of each compound and they are equal to the mass of oxygen required to full oxidize one mol of compound divided by the mass of one mol of compound.

3.2.4 Nitrogen measurement

Concentration of nitrogen in the samples was measured spectrophotometrically using the Nessler method (APHA, 1995). A calibration curve was obtained by means of standard solutions which were prepared with ammonium sulfate with concentrations ranging between 0 and 200 mgN L^{-1} in mineral medium.

Although the samples which were taken from the SBR and batch reactor were the same as the VFA samples, the procedure for the analysis diverged. Firstly, 100 µL of sample were added to 5 mL of water. Secondly, 100 µL of Nessler reagent was added and, finally, two drops of Seignette salt were added. A 15 minute waiting followed and samples were analyzed in spectrophotometer (SHIMADZU Spectophotometer UV-1800) at 410 nm. The Nessler reagent is made up of potassium iodomercurate and this compound reacts with ammonia, producing a colored complex.

3.3 Microscopic observations

3.3.1 Nile Blue staining

Intracellular PHA granules were identified using Nile Blue staining, which under epifluorescency have a red colour. As for the sampling, 100 µL of sludge, at the end of the feast phase and at the end of cycle, were

taken from the SBR and stained on a microscopy's slide. After a period for drying the biomass, it was stained with an aqueous solution of 1 % (w/v) Nile Blue at 55 °C for 10 min and washed for 1 min with acetic acid 8 % (v/v). Then, the slide was washed with distilled water and observed with epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software). Nile blue staining was applied using the method reported in the literature (Ostle & Holt 1982). This procedure was done only for the third run.

3.4 Data analysis and calculation of kinetic parameters

In order to evaluate the performance of the SBR and the batch accumulation reactor, some kinetic parameters were calculated.

In the SBR, the specific storage rate (r_{PHA}, $mgCOD_{PHA}$ $mgCOD_{X_A}^{-1}$ h^{-1}) was calculated based on the variation of the concentration of polymer (ΔPHA; mgCOD L^{-1}) between the end of the feast phase and the end of the cycle, the length of the feast phase (t, h) and the active biomass at the end of the feast phase (X_A, mgCOD L^{-1}) as follows:

$$r_{PHA} = \frac{\Delta PHA}{X_A * t}$$

The specific substrate consumption rate ($-r_s$, $mgCOD_{VFA}$ $mgCOD_{X_A}^{-1}$ h^{-1}), referring to the acids, was calculated based on the variation of the concentration of substrate (ΔS, mgCOD L^{-1}) between the beginning of the cycle and the end of the feast phase, the length of the feast phase and the active biomass. The initial value for the substrate concentration was the theoretical value corresponding to the amount of substrate fed plus the concentration present before the added substrate. As for the SBR, the last value would be zero because all substrate would have been consumed by the beginning of the cycle, which does not necessarily hold true for the batch reactor though. The equation that allows the calculation of the $-r_s$ is the following:

$$r_s = \frac{-\Delta S}{X_A * t}$$

The consumption rate of nitrogen was also calculated and the equation used was similar to the one above. However, the instant of the measured concentrations was slightly different, as the values for nitrogen considered were the ones at end of nitrogen feed and end of cycle.

While the storage yield is measurement of how much substrate is directed to polymer storage, the observed yield quantifies how much substrate was used for growth. Due to taking into consideration the biomass, it included both PHA and active biomass. Specifically, the calculated storage yield and observed yield were referred to the feast phase, by taking into account the polymer stored and VSS, respectively, in this phase. Both yields are calculated using COD units and using concentrations of polymer and biomass at the end of feast. The equations are as follows:

$$Y_{STO} = \frac{\Delta PHA}{-\Delta S} \qquad Y_{OBS} = \frac{X}{-\Delta S}$$

The OLR (gCOD L^{-1} d^{-1}) is expressed as the concentration VFA times the flow (Q, L d^{-1}) per unit of volume of the reactor (V, L):

$$OLR = \frac{S * Q}{V}$$

Finally, whenever productivity (Prod, gCOD L^{-1} d^{-1}) was calculated, it was as follows:

$$\text{Prod} = \frac{\text{PHA}_{\text{feast}} * \text{Q}}{\text{V}}$$

4 RESULTS AND DISCUSSION

As previously stated, this study aimed at investigating the influence of uncoupled feeding of carbon and nitrogen sources within the SBR cycle when compared to simultaneous feed of these two nutrients as well as the effect of COD:N ratio on the performance of the SBR operated with uncoupled carbon and nitrogen feeding. Three non-subsequent SBR runs were set up in the course of this research with different COD:N ratios. The first of those runs was performed under the exact same conditions as a SBR run from a previous research made by Valentino and colleagues, except only for the moment of nitrogen feed, which was uncoupled from the carbon feed and in this study occurred later (Valentino et al. 2014). The other two SBR runs underwent the exact same conditions as the first SBR run with the exception of the COD:N ratio. The three runs are called N-FAMINE 1, N-FAMINE 2 and N-FAMINE 3 from here on and they owe their names to the fact that nitrogen was fed during the famine phase. The first run is the one with the highest nitrogen load, whereas N-FAMINE 3 corresponds to the run with the highest COD:N ratio.

As a consequence of the similarity of results between the first two SBR runs, only one of these is thoroughly discussed, for sake of simplicity and redundancy avoidance. The second run was chosen because no batch accumulation tests were performed in the course of the first run. Therefore, the second run is explained as detailed as possible first and only afterwards the N-FAMINE 1 is mentioned. Within each run that is thoroughly discussed, the SBR performance is firstly discussed. Then, kinetic tests are discussed. This chapter is finished with the comparisons of the N-FAMINE 1 run with the work of Valentino and colleagues, referred to as N-FEAST, in which nitrogen supply was coupled to carbon feeding in correspondence to the beginning of the SBR cycle. Finally, a comparison between the three runs performed at different COD:N ratios with the SBR operated with uncoupled nitrogen and carbon feeding is discussed.

4.1 SBR run at 8.5 gCOD L^{-1} d^{-1} and 0.26 gN L^{-1} d^{-1} – N-FAMINE 2

4.1.1 SBR Performance

The MMC was fed with a synthetic mixture of acetic and propionic acids for 10 min with the purpose of achieving a FF regime leading to the selection of PHA-accumulating culture. The SBR was operated at COD:N ratio of 100:3.03 with an optimal pH value ranging between 7.5 and 7.6.

A typical DO profile is shown in Figure 4.1. This profile is indicative of substrate consumption, because when the substrates are fed to the SBR (0 min), a sudden decrease in the dissolved oxygen concentration occurs, indicating that microorganisms are metabolizing them. On the other hand, the moment there is a sudden increase in the DO concentration, it is assumed the carbon source has just finished, which means that the substrate is depleted in the reactor. Up until this moment, it is considered that the microbial cells are going through a feast phase, while after that moment, it is considered the cells are undergoing a famine phase. The decrease of oxygen concentration at minute 150 is explained by turning off the aeration during the withdrawal phase. During this run, no relevant changes in the DO were observed after the nitrogen being fed. This DO profile confirms that the culture went through both periods of feast and famine, which is a requirement for a proper selection of PHA-accumulating organisms.

The length of the feast phase during the SBR performance is reported in Figure 4.2. In the first four days, no value for the length of the feast phase is reported due to the DO profiles not revealing a clear trend for the oxygen concentration like Figure 4.1 discloses, hence hindering the determination of the length of the feast phase. This is attributed to a microbial acclimatization in the reactor, where several different microorganisms compete with each other for the substrate, because, during this period, PHA-accumulating microorganisms are not the only ones present in the reactor. Not long after this acclimatization period, this parameter

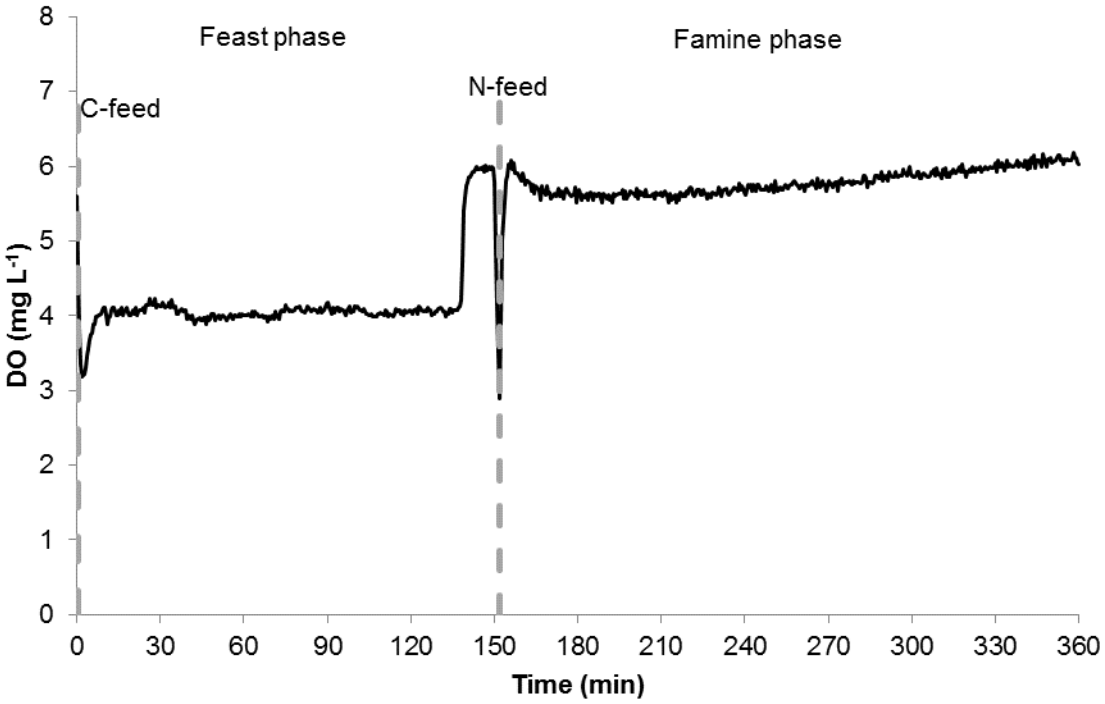

Figure 4.1 – Profile of dissolved oxygen concentration during a typical SBR cycle of the N-FAMINE 2 run.

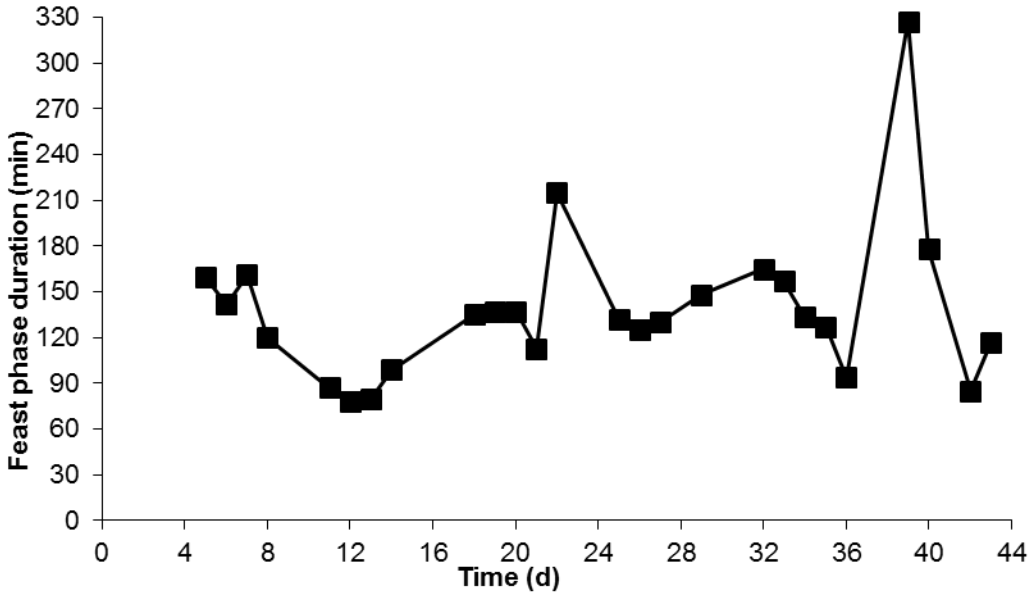

Figure 4.2 – Feast phase duration of the daily measured cycle over the SBR operation period.

with an average value of 127 ± 8 min. Some fluctuations happened during this run, leading to an unusual high length of feast phase, which can be explained by the fact that biological systems are susceptible to some variations. The feast phase period yields a ratio of length of the feast phase and length of the cycle equal to 35 ± 2 %, which according to a previous research (Dionisi et al. 2006), is above the limit (ratio of 25 %) for a good storage response, suggesting that a growth response by the cells may not have been avoided. Though,

the SBR in the previous research was operated in a different way when compared to this study, in which nitrogen supply was uncoupled from carbon feeding and occurred at the beginning of the famine phase within the SBR cycle. This difference likely affected the relation between FF ratio and storage and growth microbial response.

The concentrations of TSS and VSS over the length of the SBR cycle are reported in Figure 4.3. VSS concentration represents the concentration of biomass in the reactor. It was sampled for biomass in the moments of the SBR cycle where significant and meaningful changes could be seen, which means right before withdrawing the biomass and by the end of the cycle. Also, one extra sample was taken right before the beginning of the cycle for comparison with the end of cycle. It is worth remarking that, since this is a sequential reactor, the beginning of one cycle corresponds to the end of the previous cycle, so these two samples, in theory, should have similar values. For sake of simplicity, values corresponding to the beginning of cycle are not shown in Figure 4.3 and Figure 4.4, which reports the trends of VSS/TSS ratio and $VSS_{non\text{-}polymer}$. However, the average values of these parameters are summed up in Table 4.1.

Table 4.1 – Average values of concentration of suspended solids at three different moments of the cycle in the SBR.

	TSS (mg L^{-1})	VSS (mg L^{-1})	VSS/TSS
Beginning of cycle	2912 ± 78	2464 ± 79	0.85 ± 0.01
End of feast	3172 ± 122	2577 ± 78	0.86 ± 0.01
End of cycle	3003 ± 81	2752 ± 102	0.87 ± 0.01

Whilst in the TSS and VSS profiles the end of feast concentrations appear to be higher than both end of cycle concentrations, in the $VSS_{non\text{-}polymer}$ profile this situation does not occur so often. The difference displayed in the first two profiles relatively to the third one is caused by a higher mass of PHA by the moment carbon depletes relatively to the end of the cycles. Therefore, when VSS without the polymer is considered, the sample taken by the end of feast does not seem to have a higher concentration than the other two as represented in Figure 4.4b. Figure 4.4a shows a similar tendency as the ratio between VSS and TSS also appears to be approximately constant over time. The average values for VSS/TSS ratios can be considered as reasonably high, meaning that out of all solids present in the reactor, the majority of them were biomass, thereby favouring the process. Furthermore, a low ratio could negatively impact the PHA extraction, which in this case, should not occur considerably.

The PHA concentration obtained during the studied period has been reported in Figure 4.5. While it was sampled three times per day for biomass, five samples were taken for PHA: three in the same moments biomass was sampled and another two after end of carbon (10 min) and nitrogen (152 min) feeds. Resembling the feast phase length profile, the profile of PHA concentration shows that microorganisms underwent a short period of adaptation of eight days, as the PHA production during these days was quite low. Besides, a trend or distinction among the five samples is still not very clear. In correspondence to the 11^{th} operational day, a large PHA production occurred and continued so until the last days analyzed. After this point, a distinction between samples appears. By the end of feast phase and end of N-feed, the PHA values are clearly higher than the others, being as high as 1330 ± 73 mgCOD L^{-1} and 1229 ± 97 mgCOD L^{-1}, respectively. On the other hand, on average, the beginning of cycle, end of feed and end of cycle had the values of 545 ± 68 mgCOD L^{-1}, 503 ± 64 mgCOD L^{-1} and 628 ± 62 mgCOD L^{-1}. Based on these values, it is

possible to assume that the FF regime was successfully applied and PHA was produced during the feast phase and consumed during the famine phase.

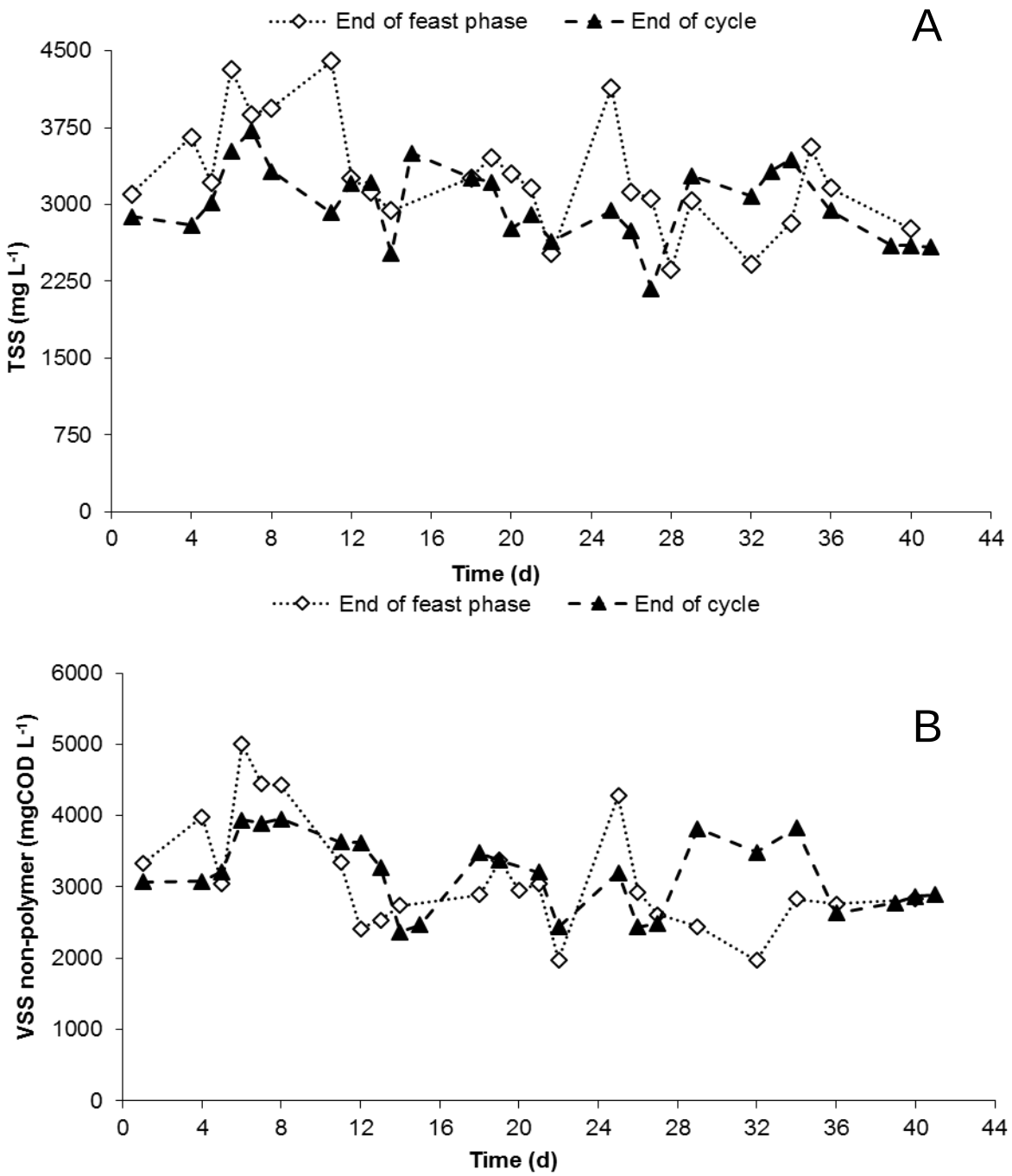

Figure 4.3 – Trend of the concentration of total (A) and volatile (B) suspended solids at different moments of the SBR cycle, during the entire period of operation of the N-FAMINE 2 run.

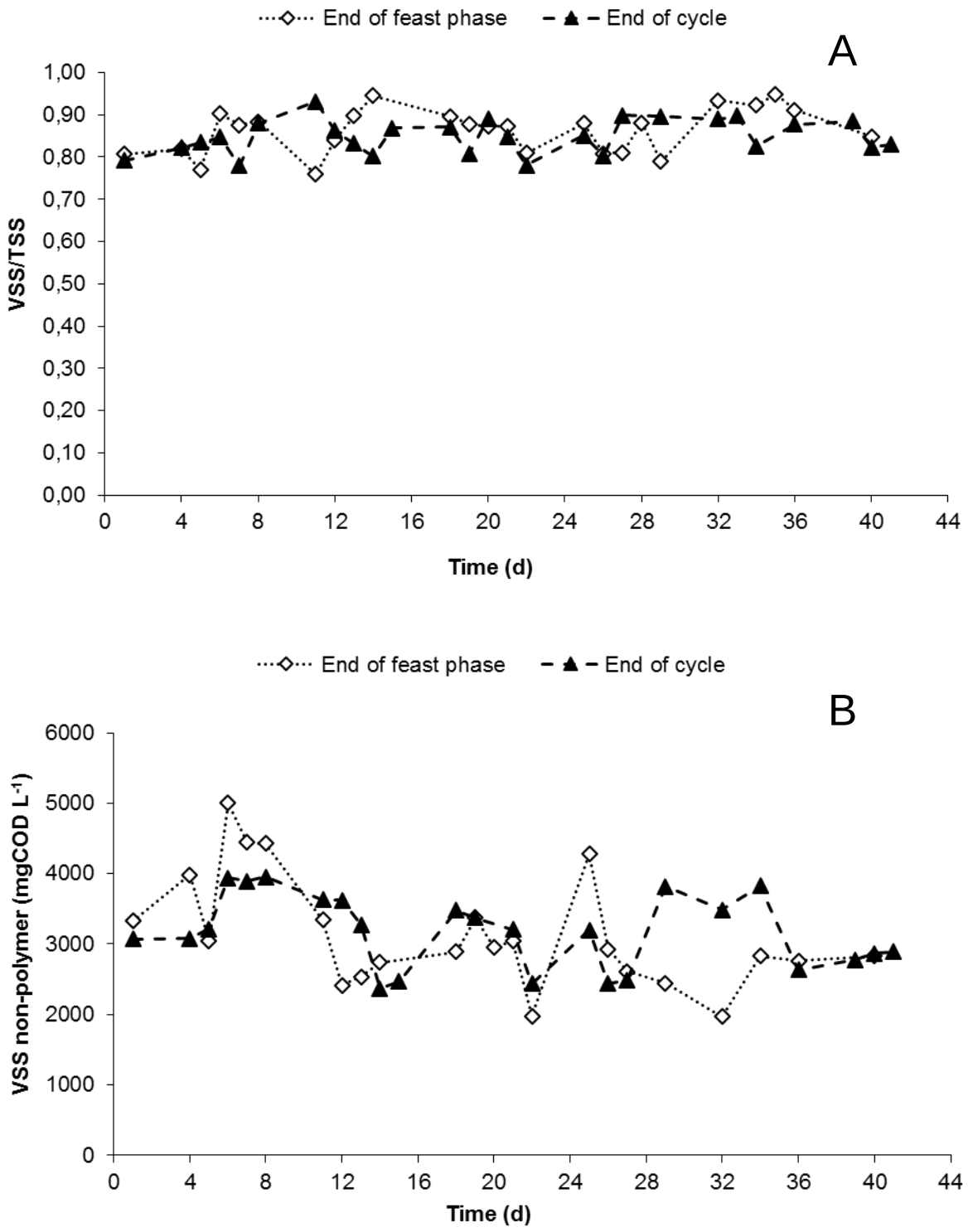

Figure 4.4 – VSS/TSS ratio (A) and non-polymer VSS (B) profiles at different moments of the SBR cycle, during the entire period of operation of the N-FAMINE 2 run.

In the 16th day of operation, more PHA was apparently produced than in the other days. This is probably not true and, these values were probably due to a technical error in the analysis.

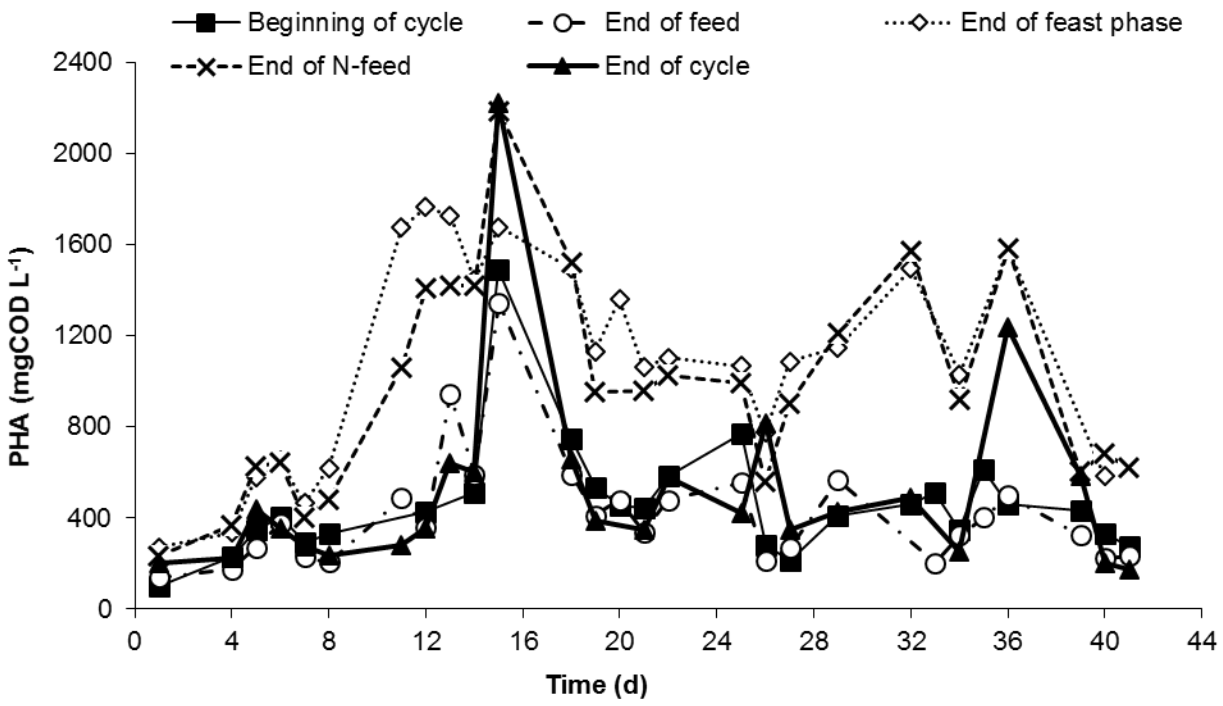

Figure 4.5 – PHA concentration at different moments of the SBR cycle, during the entire period of operation of the N-FAMINE 2 run.

Regarding the composition of the polymer, PHBV copolymer was obtained and the HV content in the polymer is represented in Figure 4.6a**.** By the start of this run, the values of HV content are quite high. A period when the percentage lowers is followed and then, these values stabilize. The reasons for this behaviour are very likely to be the same as the ones mentioned for the previous profiles. While the average values for beginning of cycle (14.0 ± 0.6 %, w/w), end of feed (14.8 ± 0.7 %, w/w) and end of cycle (14.2 ± 0.9 %, w/w) are quite similar between each other, end of feast (12.4 ± 0.5 %, w/w) end of N-feed (12.9 ± 0.5 %, w/w) are also akin. These average values were not calculated as the others before, as the values from the operational days of 25 and 26 were removed. As the end of cycle values were unusually low in these days, it is likely that some problem with the measurement occurred. The presented behaviour was unexpected, however it may be hypothesised that it was due to the presence of different microorganisms with different metabolisms, leading to a variance in the HV content. As reported in the literature, not only acetic and propionic acids may be metabolised through different pathways in different microbial cells, but also, to some extent, there is a substrate preference between different microorganisms (Dionisi et al. 2004; Serafim et al. 2008; Khanna & Srivastava 2005; Albuquerque et al. 2012; Nikodinovic-Runic et al. 2013; Yu et al. 2002). Moreover, the rate at which the monomers are polymerised into the polymer deeply depends on the enzymes present in each organism and the culture/feeding history (Laycock et al. 2014). Furthermore, degradation rates depend on monomer composition, substrate specificity and PHA depolymerase, which the last two factors depend on each organism (Yoon & Choi 1999). As a result of working with a mixed culture, different specific storage rates might have existed, as well as different HV content for each of the microorganisms. Therefore, an overall 2.4 % (w/w) reduction in the HV content, between the end of feed phase and end of feast phase, could be simply explained by the diversity of the microbial culture and its variety in metabolism. In any case, further studies should be done with the purpose of either confirm, or reject, the proposed hypothesis. Besides, it is worth mentioning that the samples beginning of cycle and end of cycle have similar values, as expected. Figure 4.6b the percentage of PHA contained in the biomass is shown. On average, a higher

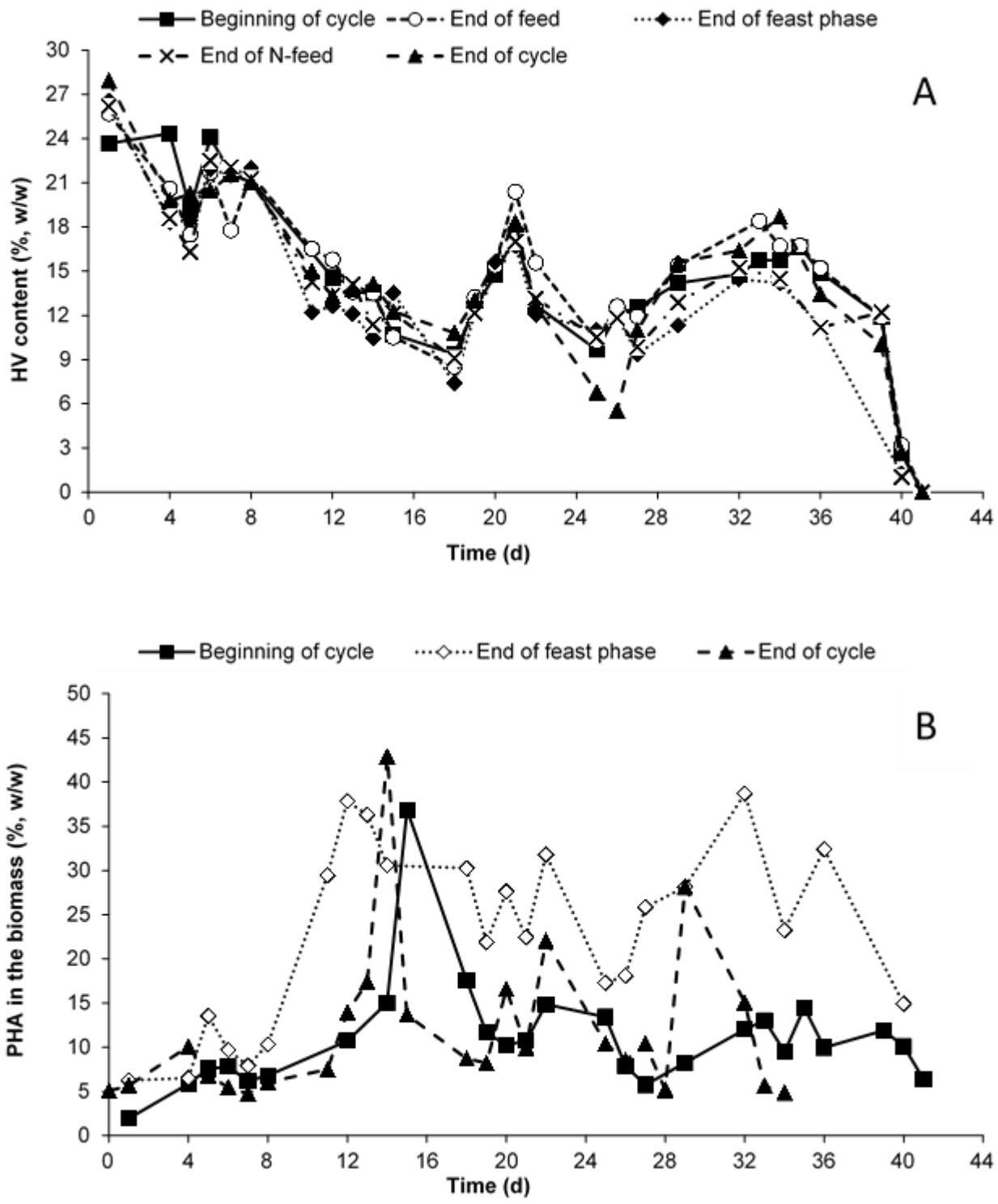

Figure 4.6 – HV content (A) and PHA content in the biomass (B) over the operational period of the SBR in N-FAMINE 2 run.

percentage of the cellular weight was due to the PHA at the feast phase (28.2 ± 1.6 %, w/w) relatively to both beginning of cycle (13.1 ± 1.7 %, w/w) and end of cycle (14.3 ± 2.4 %, w/w). This is easily explained by the

fact that the VSS did not change much over each analysed point of the cycle, but PHA did. Therefore, at the moment PHA was higher, the percentage of PHA in the biomass was also higher and vice-versa.

Figure 4.7 represents the trend of nitrogen concentration during the course of this run. Although five samples were taken in the course of this run, only three are shown here for simplicity purposes. The end of N-feed (60.7 ± 6.2 mgN L^{-1}) sample had a higher nitrogen concentration than in the other moments of the SBR cycle, as expected, because this is the instant right after the nitrogen feeding. From this moment on, cells had nitrogen available for growth. By the end of cycle (16.4 ± 4.2 mgN L^{-1}), there was still some nitrogen present which means that, while most of the nitrogen was consumed, some would still be present during the initial part of the feast phase for the microorganisms use. As a consequence of this decrease during famine phase, it means that growth occurred essentially in the course of this period, and based on the PHA profile, principally with PHA as a carbon source. However, due to leakage of nitrogen to the following cycle, some growth during feast phase could be expected. By the end of feast, the concentration was zero most of the times. Despite the representation of only 3 samples in the graphic, beginning of cycle (17.0 ± 4.2 mgN L^{-1}) and end of feed (9.8 ± 3.5 mgN L^{-1}) were also sampled but their profiles were removed for an easier understanding of the most important profiles.

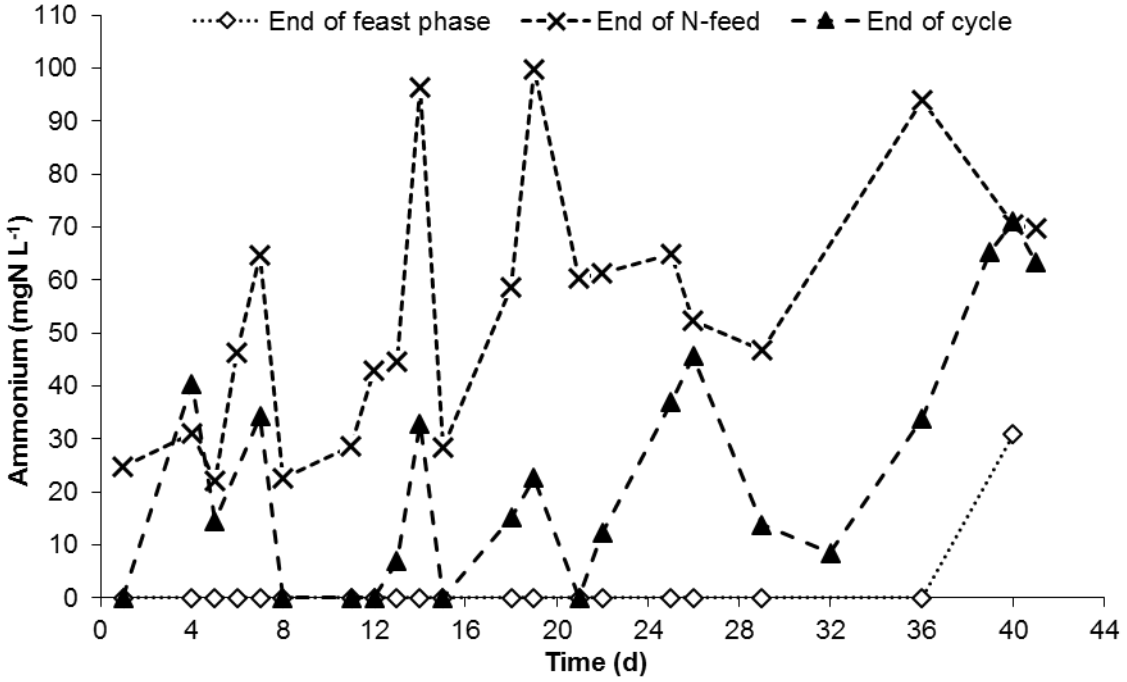

Figure 4.7 – Trend of the ammonium concentration over the operational period of the SBR.

4.1.2 SBR kinetic test

In order to understand thoroughly a typical SBR cycle, three SBR kinetic studies were performed during the course of this run. For the sake of example, the results of the first one, performed on the 20th operational day of the run, are herein described.

In the day this analysis was performed, as shown in Figure4.8a, the length of the feast phase of the SBR cycle was 137 minutes, which is correspondent to the consumption of the VFA fed at the beginning of the cycle which can also be seen in the same figure. While VFA were more concentrated in the reactor in the beginning of the cycle and were consumed during the feast phase, PHA was at its lowest value at beginning

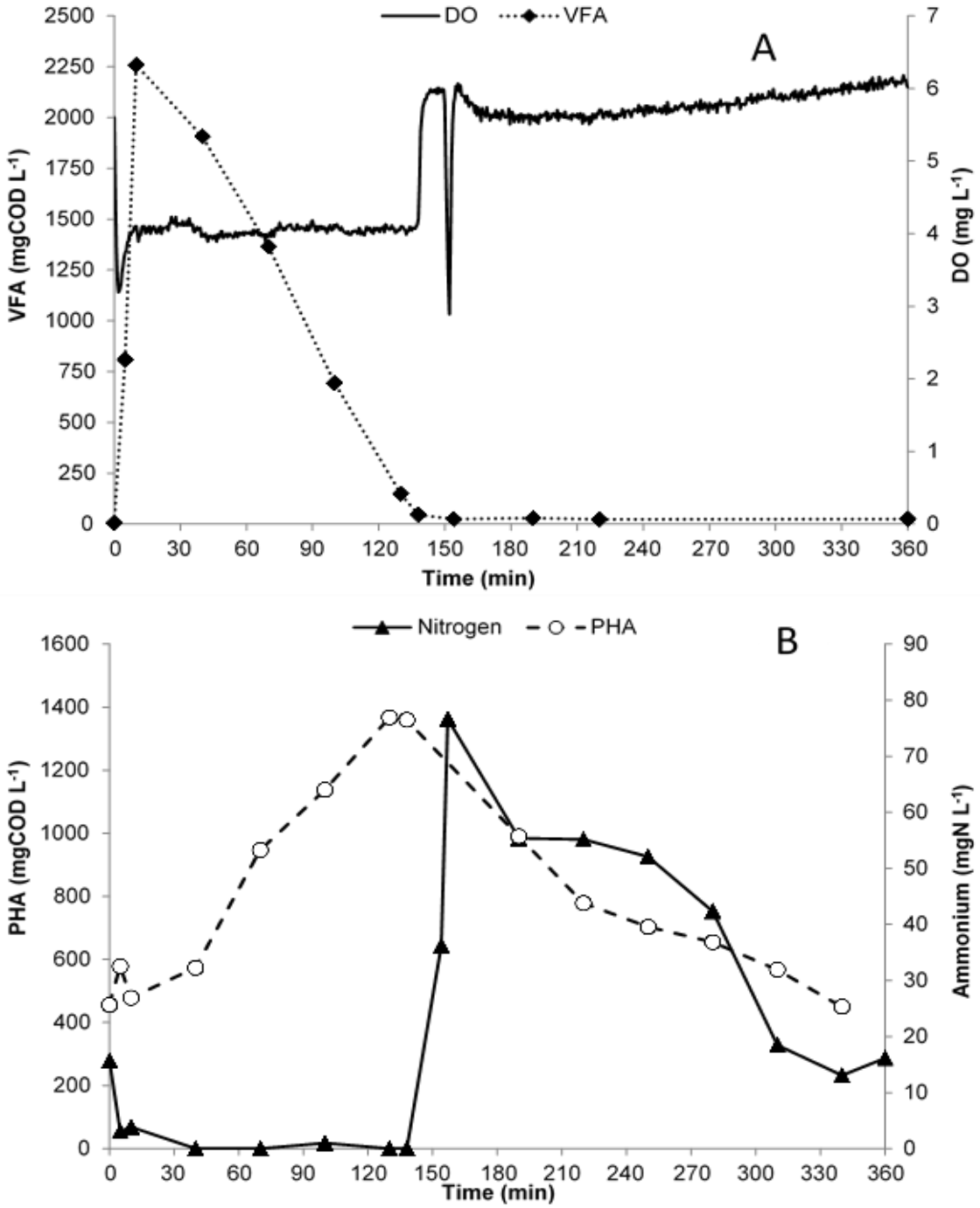

Figure 4.8 – Trend of the concentration of DO and VFA (A) and PHA and ammonium (B) during a SBR kinetic carried out when the reactor was operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and a nitrogen load of 0.26 gN L^{-1} d^{-1}.

of cycle (450 mgCOD L^{-1}), achieved its highest value by the end of feast phase (1367 mgCOD L^{-1}) and was later consumed during the famine phase achieving the lowest value, once again, by the end of cycle. VFA was consumed at a rate of 305 $mgCOD_{VFA}$ $gCOD_{XA}^{-1}$ h^{-1}, whereas PHA was formed at a rate of 133 $mgCOD_{PHA}$ $gCOD_{XA}^{-1}$ h^{-1}, which means that there was a storage yield (referred to the feast phase) of 0.43 $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$.

These profiles are a consequence of the FF regime: while VFA are abundant, cells use this carbon source for PHA accumulation as much as possible (feast phase); when the VFA are depleted, cells use the only carbon source they have in that moment (PHA) for cellular maintenance and growth, consuming it (famine phase). It is worth mentioning that PHA storage seems to be less significant in the beginning of the cycle, probably as a result of the nitrogen leakage from the previous cycle, thereby resulting in some growth. Regarding the nitrogen profile, after this nutrient was fed (258 mgN L^{-1} d^{-1}) in correspondence to the beginning of the famine phase, it was consumed over time until the end of cycle with a rate of 4.82 mgN $gCOD_{XA}^{-1}$ h^{-1}. Due to cells having presented a good storage response, which is confirmed by the PHA concentration by end of feast phase, nitrogen uptake was quite a slow process. Nonetheless, both nitrogen uptake and growth occurred during the famine phase, essentially with PHA-accumulating microorganisms, which are the only ones with abundant carbon source which may be used for microbial growth. As a consequence, by end of cycle, the nitrogen had not still been depleted, which probably happened during the first hour of the following cycle.

Profiles for PHA content in the biomass and HV content in the stored polymer are represented in Figure 4.9. The variation of the PHA content over time is similar to the one of PHA because the first variable is equal to the second divided by a constant value of $VSS_{non\text{-}polymer}$. Thus, the maximum value was achieved at the end of feast and it was 28 % (w/w). As for the HV content, the trend does not seem to be in accordance with what was previously discussed for the HV content, as the end of feast (15.6 %, w/w) has a higher value than the beginning of cycle (14.7 %, w/w). According to the trend, end of cycle would probably have had the lowest value, but the sample was lost.

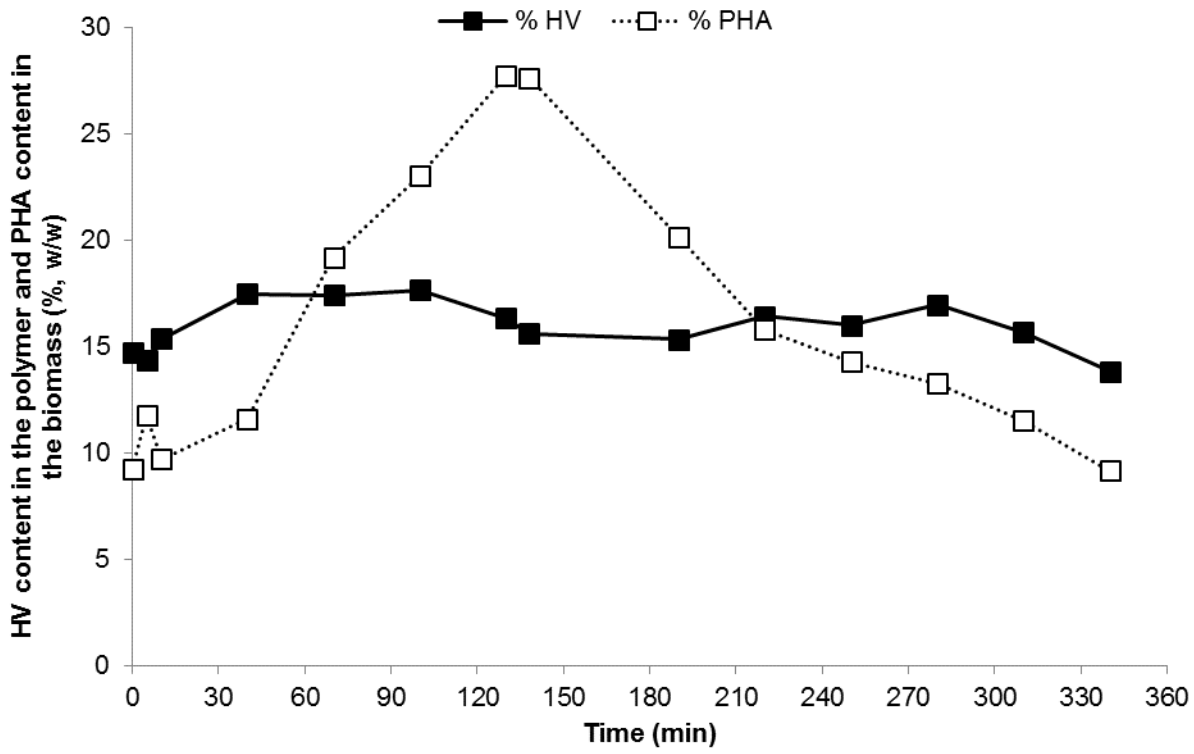

Figure 4.9 – Trend of the HV content in the stored polymer and PHA content in the biomass during a SBR kinetic carried out when the reactor was operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and nitrogen load of 0.26 gN L^{-1} d^{-1}.

Anyhow, there was some variability over the course of the run which could explain a different trend during this day.

A comparison between the 3 kinetic tests performed in the SBR during this run is summarized in Table 4.2. The last kinetic seems to be just a little different than the others because the specific PHA formation rate is the lowest (94 $mgCOD_{PHA}$ $gCOD_{XA}^{-1}$ h^{-1}), while the percentage of PHA content in the biomass is also the lowest (21.3 %, w/w). Besides, VFA consummation rate is the lowest (284 $mgCOD_{VFA}$ $gCOD_{XA}^{-1}$ h^{-1}) in this kinetic test whereas the nitrogen consumption rate is the highest (6.00 mgN $gCOD_{XA}^{-1}$ h^{-1}).

Hence, it suggests, that in this kinetic, there was a little less storage response than in the other two kinetic tests and a higher growth response. Additionally, the active biomass was higher in this kinetic test (2960 mg L^{-1}), which can explain a few parameters such as the earlier depletion of nitrogen and the percentage of polymer in the biomass. Anyhow, these kinetic tests are not very different from each other and it is very likely they give us relevant information about, for instance, the very good overall storage response present in this run.

Table 4.2 – Comparison between the 3 SBR kinetics performed in the run operated at an OLR equal to 8.5 gCOD L^{-1} d^{-1} and a nitrogen load of 0.26 gN L^{-1} d^{-1}.

	Kinetic 1	Kinetic 2	Kinetic 3	Average SBR
Day of operation (d)	20	27	34	
Feast phase (min)	138	130	133	134 ± 2,33
Maximum PHA concentration (mgCOD L^{-1})	1367	1085	1031	1161 ± 104
% PHA in the biomass of End of feast (%, w/w)	27,6	25,8	21,3	24,9 ± 1,87
% HV in the polymer of End of feast (%, w/w)	15,6	9,4	14,2	13,1 ± 1,88
Ammonium concentration by end of cycle (mgN L^{-1})	16,2	20,8	0	12,3 ± 6,31
Nitrogen consumption rate (mgN $gCOD_{XA}^{-1}$ h^{-1})	4,82	4,94	6,00	5,25 ± 0,38
PHA formation rate ($mgCOD_{PHA}$ $gCOD_{XA}^{-1}$ h^{-1})	133	139	94	122 ± 14
VFA consumption rate ($mgCOD_{VFA}$ $gCOD_{XA}^{-1}$ h^{-1})	305	376	284	322 ± 28
Storage yield (Y_{STO}^{feast}; $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$)	0,43	0,37	0,33	0,38 ± 0,03

4.1.3 Batch accumulation kinetic test

After the selection of a PHA-storing mixed microbial culture in the SBR, the PHA accumulation stage was performed. Sludge was withdrawn from the SBR in correspondence to the end of the feast phase and diluted in mineral medium (125mL of sludge in a 500 mL reactor). The mineral medium did not include any nitrogen and the accumulation tests were always performed without any nitrogen present. In this stage, fed with a synthetic mixture of acetic and propionic acids, the MMC are used with the purpose of maximizing the PHA content in the cell. Therefore, three batch accumulation tests were done in the course of this run. While the first test lasted roughly five hours, the other two lasted six hours and the feeding was variable for all of them. A very concentrated solution of a mixture of VFA (500 gCOD L^{-1}) was fed in the beginning of the test (1 mL) and once again (0.5 mL) for the first and third batches based on the oxygen uptake rate (OUR) profile. Regarding the second batch, cells were only fed in the beginning (1 mL). For the sake of example, only the accumulation test performed on the 35th day of operation of the SBR will be discussed. The OUR profile of

the batch accumulation test is represented in Figure 4.10. The consumption process of acids, is an aerobic process, which means that when the substrates are depleted, the OUR is close to none. Therefore, based on the OUR profile, an additional spike of acids was performed at 220 min and, as the VFA analysis showed, that was the moment when the substrate was depleted.

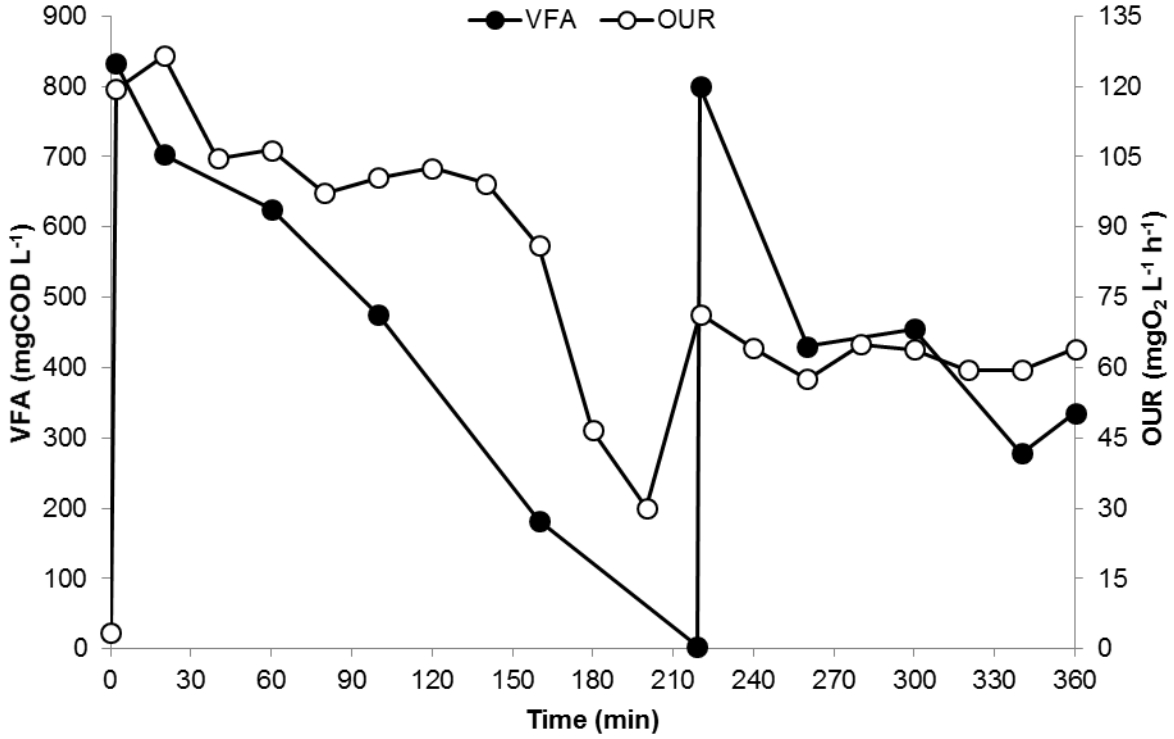

Figure 4.10 – Trend of VFA concentration and OUR throughout a typical kinetic test in the accumulation reactor with the microbial culture selected in the SBR operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and nitrogen load of 0.26 gN L^{-1} d^{-1}.

In correspondence to the first VFA spike, the OUR reached values above 100 mgO_2 L^{-1} h^{-1}, whereas after the second spike, the OUR value did not even reach 75 mgO_2 L^{-1} h^{-1}. This behaviour occurs due to a loss of the impact that the FF regime had on the cellular metabolism caused by a rather long period with VFA available. In fact, it has been reported that, after a considerably long period of carbon rich environment, storage response declines and storage occurs slower (Valentino et al. 2011). Hence, microorganisms consume VFA slower, produce considerably less PHA, and, as a result, storage capacity is lost and the saturation of the polymer inside the cells occurs (Valentino et al. 2013; Villano et al. 2014). The maximum storage capacity was reached at minute 140, which can be seen in Figure 4.11, when the maximum concentration of PHA (1057 mgCOD L^{-1}) is achieved. From that moment on, PHA decreases slightly due to lack of substrate but, after substrate is fed once again, PHA increases again and its value stabilizes. Besides, while the storage yield was 0.82 $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$ if we consider the period from minute 2 to the 160th minute, the storage yield for the overall process was 0.56 $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$. According to Figure 4.12, the maximum PHA amount yields a PHA content in the biomass of 57 % (w/w), which is a considerably high amount. Nevertheless, it is still far from 89 % (w/w), the maximum value ever achieved using MMC and a single synthetic feed of acetate (Johnson, Kleerebezem, et al. 2010). This parameter is important for the

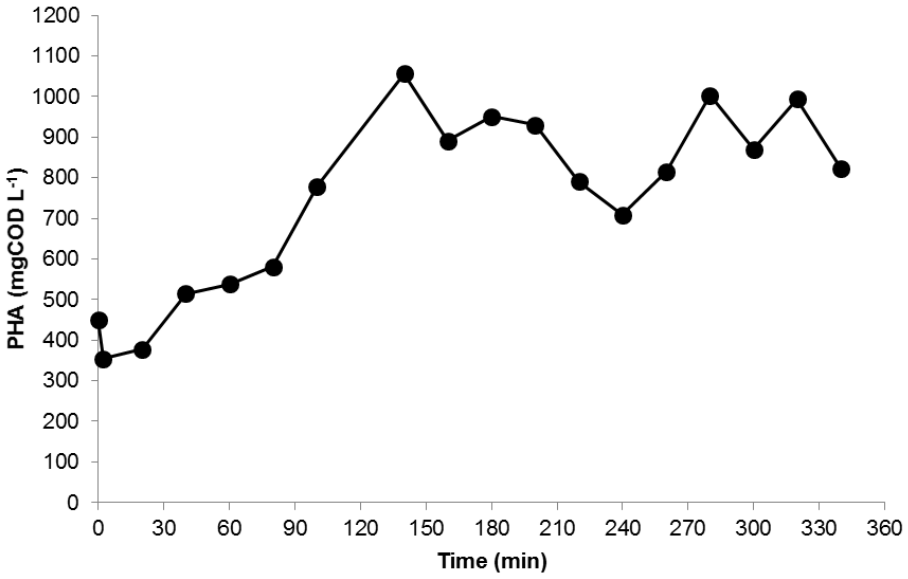

Figure 4.11 – Trend of PHA concentration throughout a typical kinetic test in the accumulation reactor with the microbial culture selected in the SBR operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and nitrogen load of 0.26 gN L^{-1} d^{-1}.

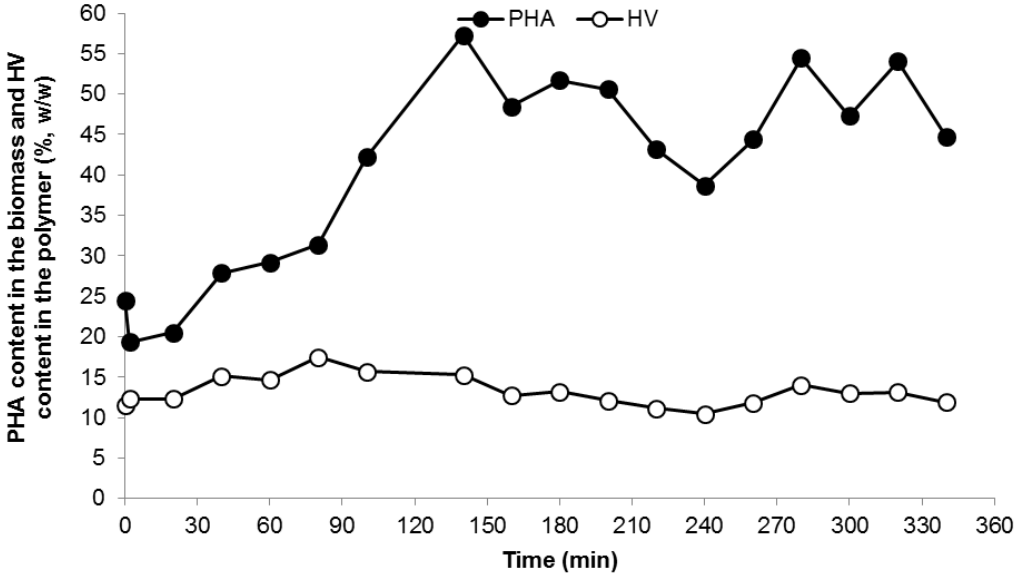

Figure 4.12 – Trend of PHA content in the biomass and HV content in the polymer throughout a typical kinetic test in the accumulation reactor with the microbial culture selected in the SBR operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and nitrogen load of 0.26 gN L^{-1} d^{-1}.

downstream costs because it may turn extraction and purification steps of the process simpler (Dias et al. 2006). Although the HV content varied considerably (10.5 % to 17.5 %, w/w), no trend stands out. This behaviour in the accumulation reactor has already been described in the literature and it should have happened due to variations in the MMC metabolism as a result of the different conditions imposed between the SBR and accumulation test (Villano et al. 2014). The differences that could have had an impact are a pH fluctuation in the accumulation reactor, the elevated concentration of carbon source and a longer period of

time with carbon available. As a result, cells had to adapt to the most recent conditions, which could have had an impact in their PHA metabolism.

A sum up of the three batch tests was done and values of the most important parameters are in Table 4.3. The values fluctuate a little, probably as a result of the performance of the previous stage of selection in SBR. Particularly, the VSS concentration directly depends on the SBR, and, as a consequence, other parameters which depend on the active biomass, for instance, the final PHA concentration, are also influenced. On the other hand, the PHA content in the biomass was not significantly affected among the three batch accumulation tests. While the HV content in the polymer is fairly alike among the three runs, the storage yield is not. Once again, it could be due to the selection previously done in the SBR, which directly affects the performance of the accumulation reactor.

Table 4.3 – Comparison between the three accumulation kinetics performed in the run operated at an OLR equal to 8.5 gCOD L^{-1} d^{-1} and a nitrogen load of 0.26 gN L^{-1} d^{-1}. Values for PHA content in the biomass and HV content in the polymer were calculated at the moment PHA was at its highest concentration. VSS values were measured at the beginning of the test.

	Kinetic 1	Kinetic 2	Kinetic 3	Average Batch
Day of operation (d)	28	33	35	
Maximum PHA concentration (mgCOD L^{-1})	470	846	1057	791 ± 172
PHA content in the biomass (%, w/w)	53,1	66,5	57,3	59,0 ± 4,0
HV content in the polymer (%, w/w)	13,9	14,5	15,3	14,6 ± 0,4
Storage yield ($gCOD_{PHA}$ $gCOD_{VFA}^{-1}$)	0,29	0,38	0,56	0,41 ± 0,1
VSS concentration at end of feast (mgCOD L^{-1})	770	1115	1274	1195 ± 80

4.2 SBR run at 8.5 gCOD L^{-1} d^{-1} and 0.32 gN L^{-1} d^{-1} – N-FAMINE 1

4.2.1 SBR Performance

This run was performed under the exact same conditions than the one previously reported, except for the nitrogen concentration. In this run, the reactor was fed with 0.32 gN L^{-1} d^{-1}, resulting in a COD:N ratio of 100:3.79.

The imposed operational conditions resulted in a length of the feast phase of 80 ± 4 min, with a correspondent length of feast phase to length of cycle ratio of 22 ± 1 %. The concentration of the active biomass was 2822 ± 121 mgCOD L^{-1}, while the PHA concentration, by end of feast phase, was 1300 ± 64 mgCOD L^{-1} and the storage yield (referred to the feast phase) was 0,40 ± 0,06 $gCOD_{PHA}$ $gCOD_{VFA}^{-1}$. Owing to the fact that microorganisms behaved similarly in these conditions relatively to the previous conditions imposed in the N-FAMINE 2 run, the conclusions that were taken from the previous run would be essentially the same for this run, therefore this run will not be further discussed. Though, a comparison between all runs performed will be made later and a table with the most important data from this run will be presented. It is worth remarking that, for the duration of this run, there were some problems with the nitrogen pump, which fed more of this compound during some days.

Nonetheless, a steady-state was achieved and the parameters were apparently not affected by this operational problem, exception only made for the nitrogen concentrations in the different moments in which samples were taken.

4.3 SBR run at 8.5 gCOD L^{-1} d^{-1} and 0.21 gN L^{-1} d^{-1} – N-FAMINE 3

4.3.1 SBR Performance

This run was performed under the same conditions as the two other runs already reported, with the exception of the nitrogen load. Indeed, 0.21 gN L^{-1} d^{-1} were fed to the reactor in course of the run, resulting in a COD:N ratio of 100:2.43.

A typical DO profile for this run is represented in Figure 4.13. This profile is fairly different from the one related to N-FAMINE 2. Firstly, the length of the feast phase is quite long, being on average 272 ± 20 min, causing a ratio of length of feast phase and length of cycle of 79 ± 5 %. Secondly, a sharp decrease in the dissolved oxygen concentration occurs by the moment nitrogen is fed. Based on the length of feast phase and length of cycle ratio, a growth response is already expected because the famine phase is not long enough to limit growth. Furthermore, due to the length of the feast phase and because the nitrogen was fed 152 minutes after the beginning of the cycle, on average, the cells had 2 hours with both nitrogen and carbon available. The sharp decrease of DO concentration after the nitrogen feed suggests an aerobic metabolism associated with growth, using both acetate and nitrogen.

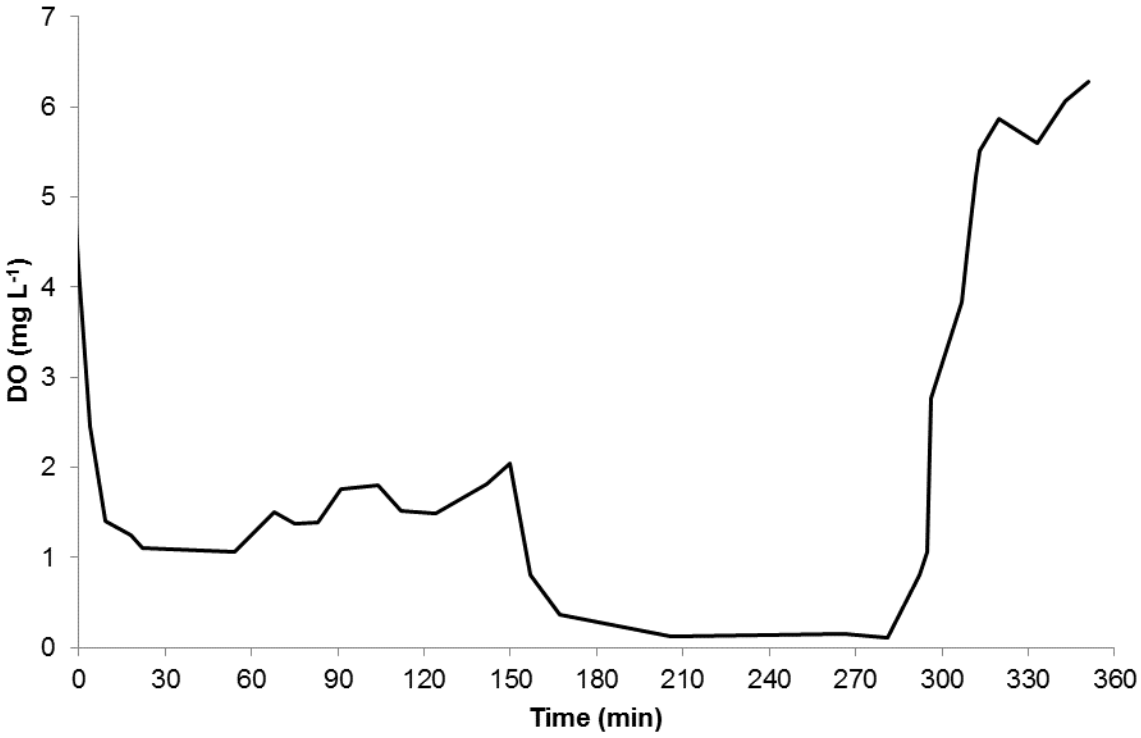

Figure 4.13 – Profile of the DO concentration during a typical SBR cycle of the N-FAMINE 3 run.

Despite the low nitrogen concentration, cells managed to grow rather well achieving a concentration of non-polymer biomass of 3596 ± 142 mgCOD L^{-1}. While the PHA by the end of feast phase was 159 ± 19 mgCOD

L^{-1}, the polymer by the end of cycle was 118 ± 20 mgCOD L^{-1}. The PHA concentrations are a result of a very poor storage ability developed in these conditions. Since the feast phase was very long, microorganisms produced either very little polymer or no polymer at all. Although the feeding contained propionic acid, the HV content was 0.98 ± 0.7 % (w/w) in correspondence to the end of the feast phase. In fact, this value is not zero because there was some HV in the first days. In contrast, during second half of the run, no HV was found in the measurements. Thus, it seems that the little polymer produced was essentially PHB and the propionic acid was used for growth and maintenance. As a result of a low polymer production, PHA content was also very low and, by end of feast phase, the average value was 3.7 ± 0.5 % (w/w).

As for nitrogen, this nutrient was readily consumed, and by end of cycle its concentration was already 9.1 ± 6.1 mgN L^{-1}. This value is not zero because in a few days some nitrogen was present, but the large majority had no nitrogen present.

4.3.2 SBR kinetic test

Two SBR kinetic studies were performed during this run with the aim of better understanding the SBR performance. For the sake of example, the results of the first one, performed on the 13th day of operation, are herein described.

VFA, PHA and N profiles are represented in Figure 4.14. While VFA were consumed over time, no clear trend of PHA formation during the SBR cycle can be seen in Figure 4.14a. The storage response was almost non-existent and, clearly, not stable during the cycle. Besides, no HV was produced during this day, in other words, all propionic acid was used for either growth or maintenance. A different hypothesis can be suggested concerning PHA storage which is that there was no PHA production and the values measured are residual and due to analytical variation. As for the nitrogen, this nutrient was quickly consumed, probably using VFA (still present after the supply of nitrogen) as a carbon source for energy. This conclusion can be made by analysing the DO profile displayed in Figure 4.13, where a drop in oxygen can be seen by the moment nitrogen is fed. This event suggests both an aerobic metabolism of the nitrogen and a higher oxygen consumption in correspondence to nitrogen feed.

All in all, the low nitrogen concentration fed to the SBR yielded a rather high active biomass concentration because of a great growth response. On the other hand, the produced biomass consumed the substrate slowly and it has never undergone a famine phase long enough to cause a good storage response.

Due to the poor SBR performance in terms of PHA production, samples of the mixed liquor were taken and analysed under the microscopic, with and without gram staining, and Nile Blue analysis were also performed. As part of collaboration with IRSA, these analyses were carried out there. Figure 4.15a shows some of the microorganisms present in the reactor after undergoing gram staining. As represented, gram positive bacillus, gram negative coccus and some fungus were present in the analysed samples. Figure 4.15b and Figure 4.15c show cells before the carbon feed and by the end of feast phase. Both samples seem not to differ much from one another regarding the total fluorescence intensity, which is in accordance with the PHA values previously reported. Despite the lack of a more specific study, the square shaped microorganisms in Figure 4.15c are probably *Lampropedia*, which tends to form those kind of structures and are a well-known PHA-accumulating microorganism (Koller et al. 2010; Beccari et al. 2009). Despite the likely presence of at least one known PHA-accumulating microorganism, under these operational conditions, the PHA production was fairly low. It might have occurred due to the long feast phase, consequently a high length of feast to length of cycle ratio was established, which allowed PHA-storing bacteria to grow well in these conditions without producing much polymer and resulting in a great growth response. Another hypothesis, though less likely, is that the PHA-storing microorganisms were not predominantly present in the reactor, resulting in poor PHA production.

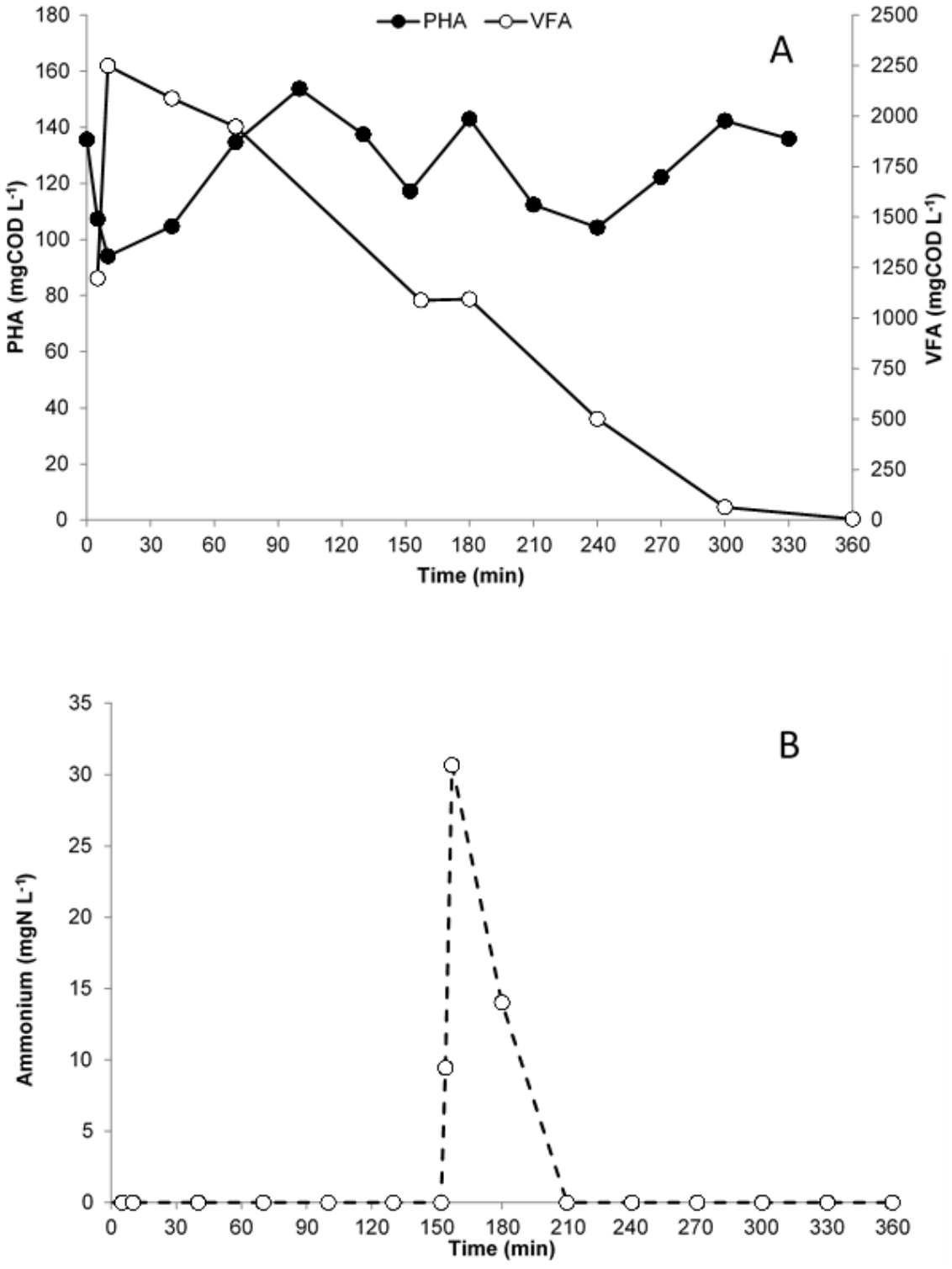

Figure 4.14 – Trend of the concentration of PHA and VFA (A) and ammonium (B) during a SBR kinetic carried out when the reactor was operated at an OLR of 8.5 gCOD L^{-1} d^{-1} and a nitrogen load equal to 0.21 gN L^{-1} d^{-1}.

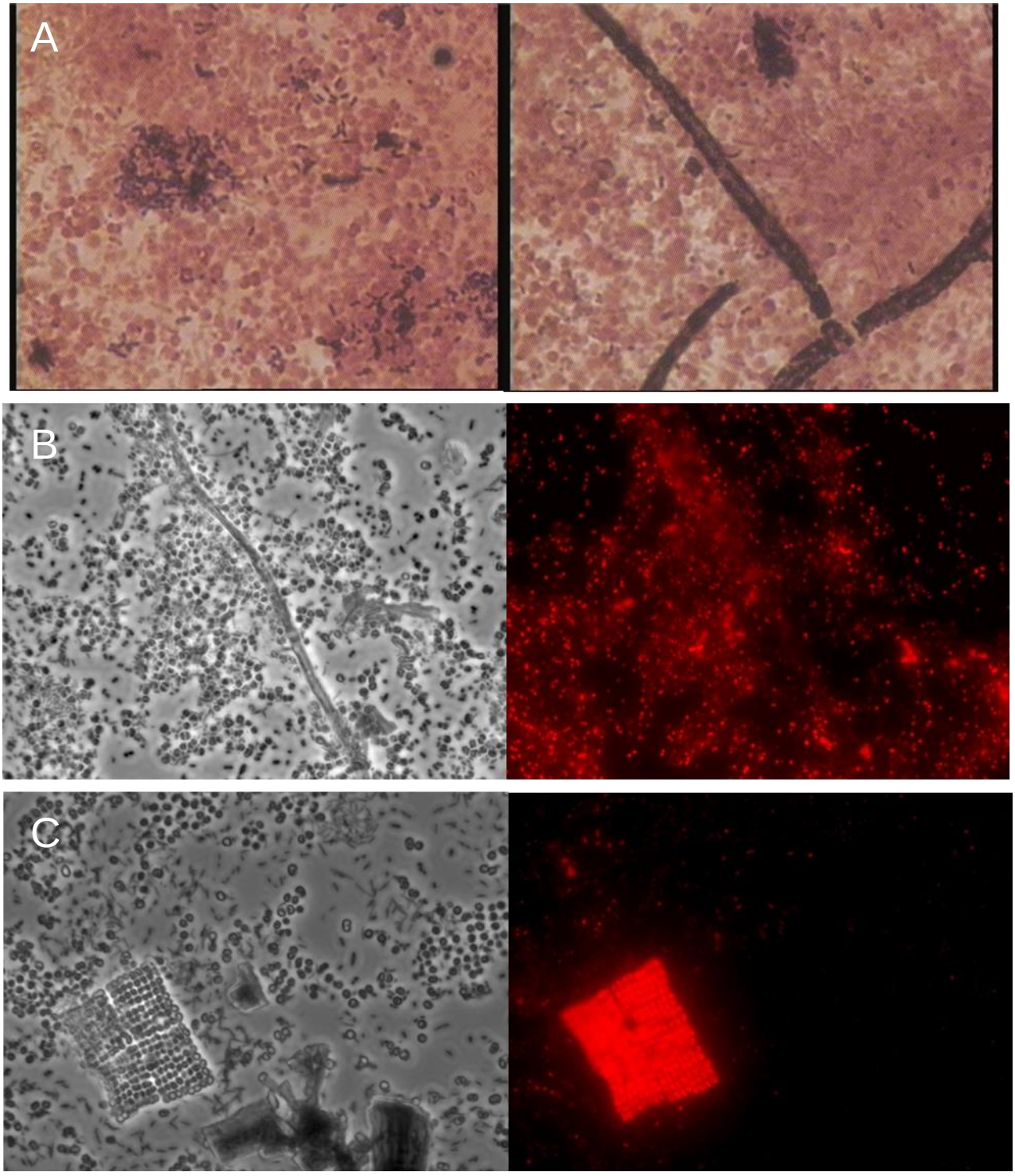

Figure 4.15 – Images taken with transmitted light, 1000x and Gram staining (A); End of cycle: phase contrast (on the left) and nile blue coloration (on the right) (B); End of feast phase: phase contrast (on the left) and Nile Blue coloration (on the right) (C).

4.4 Performance of the SBR under the different investigated operating conditions

The values of the most important parameters for each run of the SBR are summed up in Table 4.4**.** Values of a run performed prior to this study by Valentino and colleagues, with similar conditions of the ones used in this study but with coupled carbon and nitrogen feeding in correspondence to the beginning of the SBR cycle, were added to the table, for comparison purposes (Valentino et al. 2014). The values of this run are under the column N-FEAST while the values of the runs performed in this study are under the columns N-FAMINE 1 to N-FAMINE 3. Some parameters between all five runs are equal such as number of cycles per day, cycle length and OLR. The hydraulic retention time (HRT) and solid retention time (SRT) are both equal to 1 day in each run, as a consequence of not performing settling of the biomass. All the other parameters have varied for at least one of the runs.

4.4.1 Comparison of the SBR performance with coupled and uncoupled feeding of carbon and nitrogen sources

In order to evaluate the impact of the nitrogen feeding strategy on the SBR performance, results obtained in run N-FAMINE 1 have been compared to those obtained in a previous research (Valentino et al. 2014). The operational conditions of these runs are rather akin. In fact, the only difference between these runs is the moment in which the nitrogen is fed. Whereas Valentino and colleagues fed the nitrogen along with the carbon at the start of the cycle, in N-FAMINE 1 the nitrogen was fed 152 minutes after the beginning of the cycle. All parameters and nutrients' concentrations such as COD:N remained the same in these studies.

As a consequence of the different nitrogen feeding strategy, not all relevant parameters changed. Firstly, as Table 4.4 shows, the average value of the length of the feast phase was just 8 minutes longer in the run N-FAMINE 1 with respect to the run performed by Valentino and colleagues with coupled nitrogen and carbon feeding. This has an impact on the ratio of length of feast phase and length of cycle of only 2 %. As a result, the volumetric substrate rate is not affected by much as well. According to previous research, the length of feast phase to length of cycle ratio should not be higher than 20 % to 25 % in order to cells show a good storage response (Dionisi et al. 2006; Dionisi et al. 2007). This behaviour happened for both runs, suggesting not only that a good storage response occurred but also that the change of the nitrogen feed did not affect this parameter. Secondly, the non-polymer biomass and observed yield were fundamentally equal in both runs. It can be assumed that, despite the nitrogen being fed later, cells are not affected by this and grow as much as if it was fed in the beginning of the cycle. Lastly but not least, a comparable behaviour happened in the nitrogen profiles, as in both situations the nitrogen was not depleted by end of cycle. Though, in the run performed in this work, nitrogen was fed 152 minutes later, which means that it had roughly 2 hours and a half less to be consumed.

On the other hand, PHA production did not remain unchanged as a result of this modification of nitrogen feeding. In fact, the concentration of PHA by the end of feast phase increased approximately 75 %. The PHA concentration, by end of cycle, augmented even more. Owing to an equal OLR in both runs, it resulted in an increased of the storage yield as well. Besides, due to the fact that non-polymer biomass roughly did not change, the increase in PHA production caused a higher PHA content in the biomass. Likewise, an increment in the PHA productivity occurred. Importantly, even the polymer composition did not remain the same, as the HV content almost doubled. The changes in these important parameters can be attributed to the change of the nitrogen feed. Bacterial PHA synthesis is known to occur under growth limiting conditions (Albuquerque et al. 2011).

Table 4.4 – Summary of the main parameters of the SBR performed under the different investigated operating conditions.

Parameter	N-FEAST	N-FAMINE 1	N-FAMINE 2	N-FAMINE 3
Cycle per day (n)	4			
Overall cycle length (h)	6			
Volume exchanged per cycle (l)	0,25			
Ammonium feeding instant (min)	0	152		
Theoretical ammonium concentration (mgN L^{-1} $cycle^{-1}$)	79,55	80,55	64,44	51,55
Theoretical ammonium concentration (mgN L^{-1} d^{-1})	318,2	322,2	257,8	206,2
Organic load rate (gCOD L^{-1} d^{-1})	8,5	8,5	8,5	8,5
COD:N ratio (COD:gN)	100:3,74	100:3,79	100:3,03	100:2,43
Duration of the run (d)	178	51	46	21
Time needed for substrate depletion (min)	72 ± 3	80 ± 4	127 ± 8	272 ± 20
Famine phase (min)	288 ± 3	280 ± 4	233 ± 8	88 ± 20
Length of feast phase/length of cycle (%)	20 ± 1	22 ± 1	35 ± 2	79 ± 5
Nonpolymer biomass concentration at substrate depletion (mgCOD L^{-1})	2911 ± 69	2822 ± 121	2819 ± 139	3596 ± 142
Volumetric substrate removal rate (mgCOD L^{-1} h^{-1})	1822 ± 45	1647 ± 80	1069 ± 68	500 ± 43
PHA concentration at the end of cycle (mgCOD L^{-1})	197 ± 19	512 ± 77	545 ± 68	118 ± 20
PHA concentration at substrate depletion (mgCOD L^{-1})	742 ± 25	1300 ± 64	1330 ± 73	159 ± 19
Specific storage rate (mgCOD gCODnonPolym^{-1} h^{-1})	164 ± 10	201 ± 38	184 ± 34	3,34 ± 2,2
Volumetric storage rate (mgCOD L^{-1} h^{-1})	467 ± 22	589 ± 100	498 ± 90	12,4 ± 7,2
Storage yield (Y_{STO}^{feast}; gCOD $gCOD^{-1}$)	0,26 ± 0,01	0,40 ± 0,06	0,38 ± 0,05	0,02 ± 0,01
Observed yield (Y_{OBS}^{SBR}; gCOD $gCOD^{-1}$)	0,43 ± 0,01	0,44 ± 0,03	0,49 ± 0,02	0,44 ± 0,02
PHA productivity (gPHA L^{-1} d^{-1})	0,44 ± 001	0,76 ± 0,04	0,78 ± 0,05	0,09 ± 0,01
Average of ammonium concentration after N-feed (mgN L^{-1})	n.d.	117 ± 9,2	60 ± 6,2	40,6 ± 4,4
Average of ammonium concentration before end of cycle (mgN L^{-1})	34 ± 2	72 ± 6,4	17 ± 2,9	1,3 ± 1,2
PHA content in biomass at substrate depletion (%, w/w)	17,8 ± 07	28,4 ± 1,4	28,2 ± 1,6	3,7 ± 0,5
HV content in the polymer at substrate depletion (%, w/w)	10,0 ± 0,7	19,8 ± 0,9	12,3 ± 0,5	0,98 ± 0,7

In these situations, the FF regime plays an important role for PHA production to occur, leading to a growth limitation through an internal factor: insufficiency intracellular components, for instance, RNAs and enzymes crucial for growth due to extended absence of external carbon (Valentino et al. 2011; Serafim et al. 2008; Albuquerque et al. 2010). In the run performed by Valentino *et al*, this limitation had the consequence of allowing PHA-accumulating organisms to thrive because, during the famine phase, non PHA-accumulating organism had no carbon source obtainable either for growth or maintenance, thus struggling to survive. When nitrogen was fed later, not only this effect was present, but also the residual growth, that would happen for non PHA-accumulating organisms during feast phase, occurred to an even smaller extent, owing to that fact that less nitrogen was available by the beginning of the cycle. Therefore, growth occurred essentially during famine phase, while polymer accumulation during the feast phase. As a consequence, microorganisms, which did not accumulated polymer in the course of the feast phase, went through even more trouble to survive in these conditions, allowing the external carbon to be consumed by PHA-accumulating microorganisms for both growth and further PHA production during the entire cycle.

All in all, the shift in the instant of nitrogen feeding resulted in a further growth limitation, which gave rise to higher storage response, as the values in Table 4.4 of specific storage rate show. Similarly, the volumetric storage rate was higher, resulting in an overall improvement of the MMC selection in the SBR.

4.4.2 Effect of the COD:N ratio on the SBR performance with uncoupled carbon and nitrogen feeding

These three runs (N-FAMINE 1, N-FAMINE 2 and N-FAMINE 3) were operated in pretty similar conditions. Indeed, the nitrogen load is the only operational parameter that was changed from run to run. From the first run to the second, there was a 20 % decrease in the nitrogen amount given to the reactor as well as another 20 % decrease from the second run to the third one. These variations resulted in the supply of 80.55 mgN L^{-1} $cycle^{-1}$, 64.44 mgN L^{-1} $cycle^{-1}$ and 51.55 mgN L^{-1} $cycle^{-1}$ to the first, second and third runs, respectively. Such variations in the nitrogen provided to the reactor, caused the COD:N to vary, leading to values of 100:3.79, 100:3.03 and 100:2.43, respectively. All other parameters remained unchanged, including the nitrogen feed, which occurred at 152 minutes from the beginning of the SBR cycle. The only exception made was for the withdrawal of the mixed liquor in the N-FAMINE 3. In the course of this run, when it was noticed that the feast phase was longer than 150 minutes, the withdrawal was changed to right before the end of the cycle so that carbon would not be lost in the process.

Figure 4.16 shows how the feast phase was influenced by the COD:N ratio. A change in the cellular metabolism occurred as a result of the decrease in the nitrogen concentration. More specifically, a nitrogen reduction led to a slower catabolism of the substrate, resulting in a longer feast phase. An unexpected behaviour occurred in growth, as according to Figure 4.16, either non-polymer biomass was not affected by a decrease in nitrogen (from COD:N ratio of 100:3.79 to 100:3.03) or cells managed to grow more with even less nitrogen (from COD:N ratio equal to 100:3.03 to 100:2.43). This suggests that a decrease in the nitrogen concentration did not cause growth impairment, but affected the cellular metabolism instead. It is very likely that biomolecules which are made out of the nitrogen, such as proteins and RNA, were in progressively smaller amount as COD:N ratio incresed. The affected proteins were the ones that did not carry out any vital function to the cell, allowing cells to grow fairly well with different nitrogen concentrations. Some of these proteins were probably associated with metabolism, explaining why the feast phase became longer as the nitrogen load was shorter.

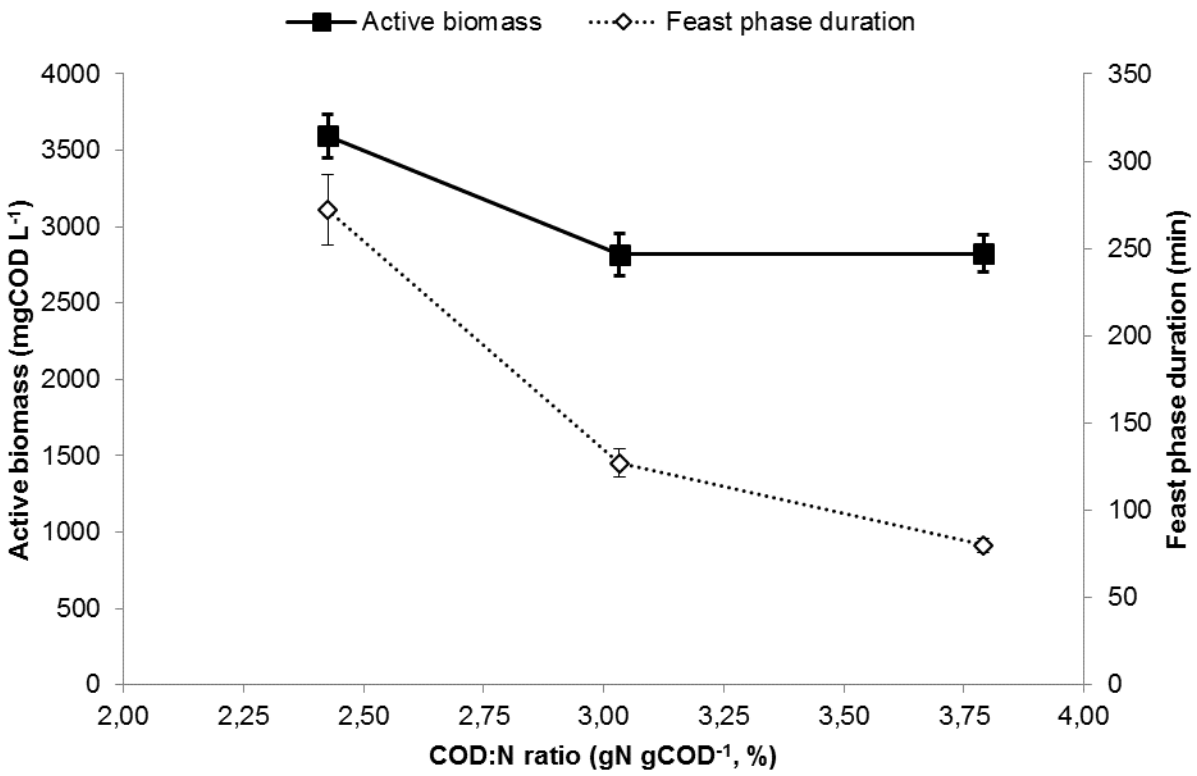

Figure 4.16 – Averages values of feast phase duration and active biomass as a function of the COD:N ratio.

The trends of PHA concentration and HV content in correspondence to the end of feast are represented in Figure 4.17. The runs with the two lower COD:N ratio achieved an average maximum PHA concentration by end of feast phase rather similar, whereas the run with the highest investigated COD:N almost did not produce any PHA. As for the HV content, it decreased as COD:N ratio increased. As reported in the literature, not only COD:N ratio has been known to affect the monomer composition, but also, under nutrient stress from nitrogen and phosphorus, HV content tends to decrease (Wen et al. 2010; Hong et al. 2000). The change in polymer composition between the two lowest investigated COD:N ratios, suggests a difference in polymer formation and PHA metabolism despite an overall productivity quite similar. Regardless the reasons for this behaviour, it means that regardless the composition of the feedstock, for a narrow range of COD:N ratios, polymer composition can be tailored just by a small shift in this parameter without a significant loss in productivity. Since HV content has a significant impact on the final polymer properties, the industrial application of this discovery is definitely noteworthy (Arcos-Hernández et al. 2013; Salehizadeh & Van Loosdrecht 2004; Reis et al. 2003). The storage yield for each run is represented in Figure 4.18 and it was similar for the lowest investigated COD:N ratios whereas substantially decreased at the highest investigated ratio. The behaviour presented by the microorganisms under the lowest nitrogen concentration was quite distinct from the behaviour under the other nitrogen concentrations. Under the most limiting conditions, a global growth response occurred, which means that, despite cells having had less nitrogen available, they grew more anyway. This was a result of a pretty short famine phase, which was not long enough to cause an effective FF regime. As a consequence, microorganisms barely produced PHA and grew as much as the conditions let them. Furthermore, according to literature, the SRT may have also played a role here, as nitrogen limitation and a low SRT tend to adversely affect the PHA production (Chang et al. 2012). On the other hand, the famine phase was long enough in the two less nitrogen limiting conditions in order to avoid a growth response. As a consequence, cells manage to store polymer efficiently.

One, yet unexplained, big difference that was noticeable and it was not quantified, unlike the other parameters, is the viscosity of the liquid inside the reactor. In the run with the lowest nitrogen concentration, the fluid was definitely more viscous than in the other runs, having the appearance of a gel. It has been previously shown that viscous liquids as a result of microorganisms' activity may appear as a result of exopolysaccharides production (Zhao et al. 2013). According to Figure 4.15, N-FAMINE 3 had fungi present and it has been known for quite some time that many of these organisms have the ability to produce a wide variety of exopolysaccharides (EPS) under limitation or even exhaustion of nitrogen (Seviour et al. 1992; Vijayendra & Shamala 2013). These organisms produce EPS as a strategy to protect themselves against phagocytosis and from adverse conditions, to enhance growth and as a mean to adhere to a surface, among others (Poli et al. 2011; Weiner 1997). Hence, it is likely that, with a very high COD:N ratio, fungi grew quite well, produced EPS and it resulted in a more viscous environment. Despite the lack of the determination of the cultures present in N-FAMINE 1 and N-FAMINE 2, it is quite safe to assume fungi were not present, due to various reasons such as the apparent low viscosity of the liquid and the high PHA production that is never tightly associated with fungi presence.

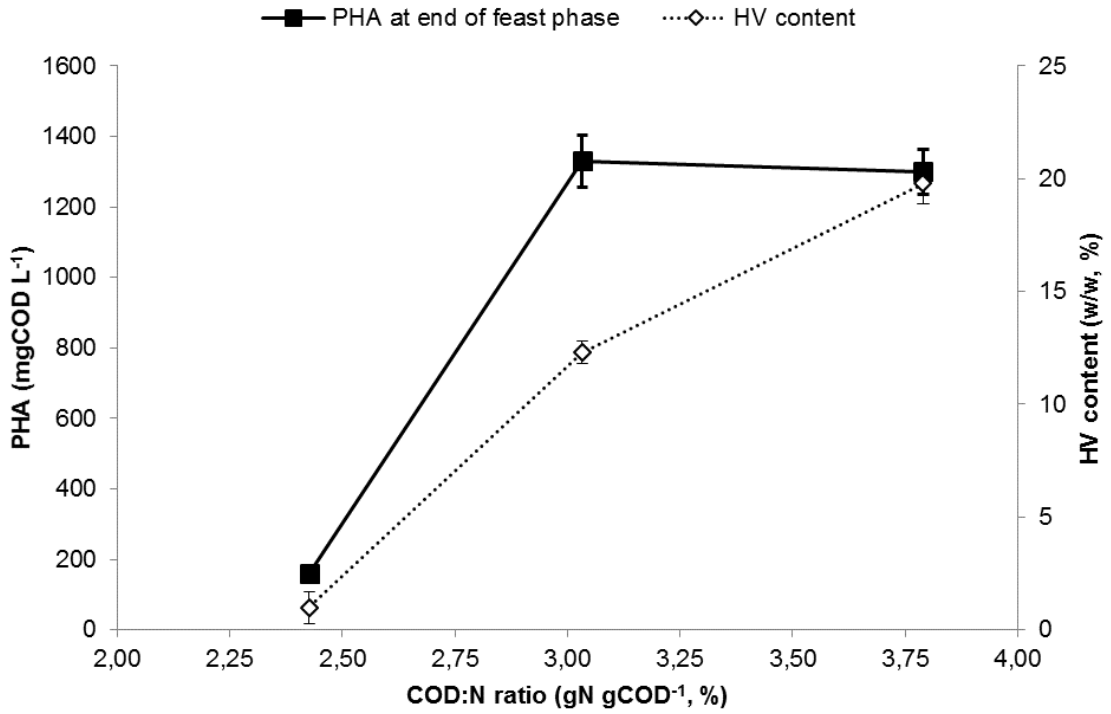

Figure 4.17 – PHA concentration at the end of the feast phase and HV content in the polymer as a function of the COD:N ratio.

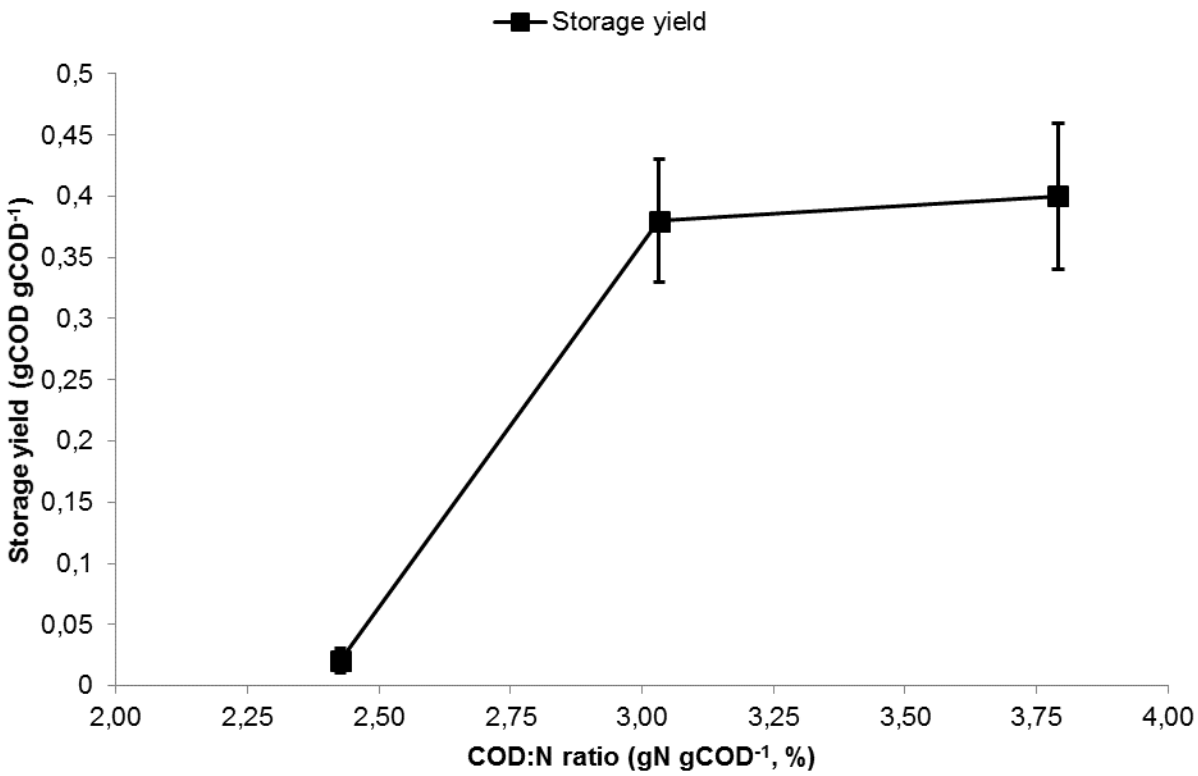

Figure 4.18 – Storage yield as a function of the COD:N ratio.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, PHA production was evaluated by means of MMC using a synthetic mixture of acids, at a high OLR (8.5 gCOD L^{-1} d^{-1}) and variable nitrogen concentrations through three different runs. Although the three runs were performed with similar conditions among each other, the nitrogen load varied. The SBR runs, which for the purpose of this work were denominated as N-FAMINE 1, N-FAMINE 2 and N-FAMINE 3, were performed with COD:N ratios of 100:3.79, 100:3.03 and 100:2.43, respectively. Besides, nitrogen was not fed along with the carbon source, being fed only during the famine phase. N-FAMINE 1 was performed under similar conditions as the run performed in a previous research (referred to as N-FEAST), except for the nitrogen feeding (Valentino et al. 2014). While Valentino and colleagues used a coupled feeding strategy of carbon and nitrogen sources, in this study, in each SBR run an uncoupled strategy of nitrogen supply from carbon feeding was adopted. Therefore, the impact of the nitrogen feeding strategy on the SBR performance as well as the influence of the COD:N ratio (with uncoupled carbon and nitrogen feeding) on the SBR operation have been assessed.

Firstly, as discussed in the previous section, the N-FEAST run performed by Valentino and colleagues was similar to N-FAMINE 1, with an exception made for the instant when the nitrogen was fed. Albeit most kinetic parameters remained fairly similar, the imposed change led, not only to a substantial increase in the PHA production (up to 1.3gCOD L^{-1}), but also to a faster polymer production by the MMC. This clearly indicated that uncoupling nitrogen supply from the carbon feed in the SBR enhances the selective pressure towards PHA-storing microorganisms. Although this evidence has been demonstrated only for the conditions used, it is a good sign that a later feed of nitrogen seems to induce a stronger storage response.

Secondly, it can be concluded that nitrogen concentration plays a critical role on the SBR performance, considering the studied conditions. While the two lowest investigated COD:N ratios (100:3.79 and 100:3.03) yielded good microbial storage response, the lowest investigated ratio (100:2.43) resulted in a very long feast phase (272 ± 20 min) with a consequent short famine phase (88 ± 20 min), indicating that the FF regime required for the selection of the PHA-storing microorganisms was not well established in the SBR. This led to the predominance of a growth response over storage response. In fact, despite the lowest nitrogen concentration, in this run the non-polymer biomass was the highest among the three runs. In agreement with this evidence, it was found that fungi were a reasonable part of the living organisms in the reactor, leading to a high fluid viscosity. In the course of this study, it was not measured the point or range at which there is a sharp decrease or unstable performance, respectively, in SBR performance as a result of lowering the nitrogen concentration. However, taking into consideration the obtained results, it is possible that between the COD:N ratios of 100:3.03 and 100:2.43, there is a point or range at which it occurs. Furthermore, it seems that there is a correlation between the COD:N and the HV content. A decrease in the nitrogen feed led to a decrease in the HV content in the polymer (from 19.8 ± 0.9 % (w/w) to 0.98 ± 0.7 % (w/w)), in spite of the fact the carbon feedstock composition remained unchanged. The underlying cause for this result remains unknown though. In the case of the COD:N ratios of 100:3.79 and 100:3.03, since the performances were rather identical, it can be assumed that this parameter can be used to adapt the final PHA composition to fit the final polymer usage, with negligent loss of performance. However, in order to take advantage of this property, further studies should be done in order to understand how HV content varies for other COD:N ratios, and how other parameters such as SRT, OLR, feedstock composition or cycle length can affect how COD:N ratio leads to a higher or lower HV content.

Last but not least, it seems that the general idea of existing a requirement for a feast length to cycle length ratio lower than 25 %, in order to result in a good selection does not necessarily apply. Indeed, the N-FAMINE 2 run resulted in a performance as good as the N-FAMINE 1, and reasonably better than the N-FEAST, as the PHA at the end of feast (1330 ± 73 mgCOD L^{-1}) and storage rate (184 ± 34 $mgCOD_{PHA}$ $gCOD_{XA}^{-1}$ h^{-1}) values show. However, in that run, the ratio of feast length and cycle length was higher than 25

% (accounting for 35 ± 2 %). This conclusion becomes interesting if it is taken into consideration that there are factors, for instance the OLR, that significantly contribute to increase the ratio of feast length to cycle length. Therefore, in theory, there is a chance that the SBR performance can be further improved using a higher OLR while limiting growth by other means than a long famine, such as nutrient limitation.

Despite the fact that an optimization to a previous work was achieved, there is still a long way to go in what concerns PHA production using MMC. Firstly, since PHA production studies with MMC are aimed at the production using wastewaters, a step forward could be trying the exact same conditions using wastewaters and try to understand if the storage response remains unaffected. Secondly, studying different conditions with a synthetic mixture can still be a good opportunity to try an improvement before the use of residual wastewaters. One approach could be using the lowest investigated COD:N ratio at a higher OLR to check if, despite an obvious increase in ratio of length of feast phase to length cycle, the PHA at the end of feast phase increases even more. Therefore, upon further optimization, the PHA production process by MMC could be potentially simplified by directly sending the biomass withdrawn from the SBR (rich in polymer) to PHA downstream processing with no need for an intermediate accumulation stage. A better understanding of how COD:N can interfere with the PHA production metabolism and how it results in a lower/higher HV content, might come in handy when the process advances to an industrial production process. In what regards the global process of PHA production, it will definitely be critical to optimise the PHA extraction process in order to make it more as much eco-friendly as it can become, as well as economically competitive.

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