Xiaofei Wang

Mestre em Ciências

Impact of dissolved oxygen on PHA production and integration with biological nutrient removal processes

Dissertação para obtenção do Grau de Doutor em
Química Sustentável

Orientador: Maria da Ascensão Carvalho Fernandes de Miranda Reis
Professora Catedrática
Faculdade de Ciências e Tecnologia
Universidade Nova de Lisboa

Co-orientadores: Gilda de Sousa Carvalho
Investigadora Principal
Faculdade de Ciências e Tecnologia
Universidade Nova de Lisboa

Adrian Michael Oehmen
Professor Auxiliar Convidado
Faculdade de Ciências e Tecnologia
Universidade Nova de Lisboa

Júri:

Presidente:	Prof. Doutor Fernando José Pires Santana
Arguentes:	Prof. Doutora Maria Catarina Marques Dias de Almeida Prof. Doutor Bruno Sommer Ferreira
Vogais:	Prof. Doutora Maria da Ascensão Carvalho Fernandes de Miranda Reis Doutor Pedro Ricardo Neto Póvoa Doutora Catarina Silva Simão de Oliveira

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

April, 2018

Impact of dissolved oxygen on PHA production and integration with biological nutrient removal processes

Acknowledgements

When I finally stand at the finishing line of my PhD and look back to the road that I have traveled along in the past four years, I feel nothing but grateful.

First of all, I own my gratitude to the EU, for the financial support from MERMAID project "Microbial Resource Management and Engineering for Closing the Urban Water Cycle", (a Marie Skłodowska-Curie Initial Training Network, under grant number 607492). I am so lucky to get this opportunity to conduct my work in the amazing Portugal.

Professor Maria Ascensão Reis, Professor Adrian Oehmen, and Doctor Gilda Carvalho have been my greatest allies through the entire PhD. Without your supervision, I have no chance to finish the thesis. São, I will always remember how dedicated you are to the research, and how kind you are to us students. You are my life model to be a strong, dedicated, and independent woman. Adrian, you must have some magic power to break obstacles. After every single meeting with you, I felt so much inspired and motivated. Gilda, you are not only an excellent supervisor to me, but also a big sister who cares about me and always makes me feel better at those fragile moments. It was a great honor to work with you all.

I would like to give my special gratitude to Doctor Simon Bengtsson and Professor Alan Werker. The 4 months working with you in Lund was such a pleasure. Thanks for the suggestions and discussions from experimental plan to data analysis, the hands-on supervision from the lab to the field, and the friendship in the chill Nordic winter. I am also grateful to other people in AnoxKaldnes and Bath WWTP, especially Anton Karlsson for the analysis, and Knees and Martin for the sampling and the pickups.

To all the colleagues in the Bioeng group, it has been such a nice experience to work with you all. I enjoyed a lot from the monthly meeting, the picnics, and the continuous friendship that you have shared with me. Special thanks to Elsa Mora and Mónica Centeio, for your kind help of the urgent analysis, and the office mates in 602, for the company and encouragement all the time.

To my sweet flat mates, thanks for everything in the house and outside. Virginia, you are an angel in solving all the Portuguese related problems. Yen, your Pho is the best that I have ever tasted, I will make sure to pay you a visit in Vietnam!

To all the members in the Mermaid projects, and all the friends in Portugal and anywhere else, you are important components of this wonderful journey.

Last but not least, to my family: 我要感谢我的家人，我的爸爸妈妈，爷爷奶奶，姥姥，还有永远在我心中的姥爷，虽然身在千里之外，我每时每刻都能感到你们的关心和支持，我会好好加油，成为让你们骄傲的人。最后的最后，我要感谢我最好的朋友和伴侣，吴晨，谢谢你的爱与鼓励，让我有机会成为与你比肩的橡树。

Abstract

Bio-based and biodegradable polymers are regarded as potential replacements of traditional fossil based plastics. Polyhydroxyalkanoates (PHAs) are widely studied biopolymers due to their broad range of thermal and mechanical properties. The impacts of operational conditions, such as pH, temperature, organic loading rate and sludge retention time, in PHA production process by mixed microbial cultures (MMCs) have been evaluated by previous studies, but the influence of dissolved oxygen (DO), which is directly related to process energetic demand, was rarely discussed. This thesis is focused on the effects of DO on the microbial culture selection and PHA accumulation stages, as well as the impact of DO on a combined PHA production and nutrient removal process for wastewater treatment. Efficient *Plasticicumulans* dominating microbial cultures were enriched under both high DO (3.47 ± 1.12 mg/L) and low DO (0.86 ± 0.50 mg/L) conditions in the feast phase containing mostly the same populations but with different relative abundance. Butyrate and valerate were found to be the preferred substrates as compared to acetate and propionate regardless of DO concentrations. Compared to acetate and propionate, the butyrate and valerate uptakes were not significantly impacted by low DO levels in the PHA accumulation stage. A metabolic model was developed for the first time to describe the substrate preference among multiple volatile fatty acids (VFAs), providing a successful approach for PHA composition prediction and process efficiency optimization when four competing VFAs are supplied. Further, DO level control through both the feast and famine phases of culture selection was applied, at 3.79±0.65 mg/L and 0.48±0.29 mg/L respectively. The low DO level in both the feast and famine phases proved to be insufficient for successful MMC enrichment. By characterizing the microbial evolution under the unlimited DO conditions, it was found that *Paracoccus* was the dominating population (>50%) in the selected cultures, and substrate competition was correlated with the abundance of *Plasticicumulans* during culture selection. In order to optimize the integrated PHA production and nutrient removal process fed with ammonia-rich feedstocks in full scale implementation, the impact of DO on both processes was investigated. Much higher DO affinity for VFA consumption was observed as compared to nitrification. A DO control strategy was proposed based on the observation that the PHA production was not influenced while nitrogen was removed by simultaneous nitrification and denitrification processes when controlling DO at low levels (e.g. 0.4-0.8 mg/L).

Keywords: polyhydroxyalkanoates (PHAs), mixed microbial cultures (MMCs), dissolved oxygen (DO), substrate competition, metabolic modeling, nitrification.

Resumo

Polímeros biodegradáveis de base biológica são considerados como um potencial substituto de plásticos tradicionais com base fóssil. Polihidroxialcanoatos (PHAs) são biopolímeros vastamente estudados devido à sua variada composição, possuindo uma ampla gama de propriedades térmicas e mecânicas. O impacto das condições operacionais, tais como pH, temperatura, carga orgânica, e tempo de retenção de lamas, no processo de produção de PHA por culturas microbianas mistas (MMCs) foram avaliados em estudos anteriores, mas a influência do oxigénio dissolvido (DO), que está diretamente relacionado com os requisitos energéticos do processo, discutiu-se raramente. Esta tese está focada no efeito de DO nas fases de seleção da cultura microbiana e de acumulação de PHA, assim como no impacto do DO num processo de tratamento de águas residuais combinando a produção de PHA com a remoção de nutrientes. Culturas microbianas dominadas pelo eficiente organismo *Plasticicumulans* foram enriquecidas sob condições tanto de alto DO (3,47 ± 1,12 mg/L) como baixo (0,86 ± 0,50 mg/L) na fase de fartura, contendo principalmente as mesmas populações mas com diferente abundância relativa. Observou-se que butirato e valerato são os substratos preferenciais em comparação com acetato e propionato independentemente da concentração de DO. Em comparação com acetato e propionato, os consumos de butirato e valerato não foram significativamente impactados por baixos níveis de DO na fase de acumulação de PHA. Foi desenvolvido um modelo metabólico que, pela primeira vez, descreve a preferência de substrato entre múltiplos ácidos gordos voláteis (VFAs), fornecendo uma abordagem bem-sucedida para a previsão da composição de PHA e a otimização da eficiência do processo quando quatro VFAs competidores são fornecidos. Posteriormente, o DO foi controlado durante os períodos de fartura e de fome da fase de seleção da cultura, a 3,79±0,65 mg/L e a 0,48±0,29 mg/L. O nível limitante de DO provou ser insuficiente para enriquecimento de uma MMC acumuladora de PHA. A caracterização da evolução microbiana nas condições de DO não limitantes, revelou que *Paracoccus* era a população dominante (> 50%) nas culturas selecionadas, e competição entre substratos foi correlacionada com a abundância de *Plasticicumulans* durante a fase de seleção. A fim de otimizar na implementação a escala real da produção de PHA integrada com o processo de remoção de nutrients, alimentados com matérias-primas ricas em amónia, o impacto do DO em ambos os processos foi investigado. Observou-se uma afinidade de consumo de VFA muito maior em comparação com a nitrificação. A estratégia de control de DO foi proposta, com base na observação de que a produção de PHA não foi influenciada enquanto o azoto foi removido por processos de nitrificação e desnitrificação simultâneos quando se controlarou o DO a níveis baixos (ex. 0,4-0,8 mg/L).

Palavras-chave: Polihidroxialcanoatos (PHAs), culturas microbianas mistas (MMCs), oxigenio dissolvido (DO), competição de substrato, modelação metabólica, nitrificação.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

ATP	Adenosine triphosphate
AOB	Ammonia oxidizing bacteria
BNR	Biological nutrient removal
COD	Chemical oxygen demand
DO	Dissolved oxygen
EPS	Exopolysaccharides
F/M ratio	Food to microorganism ratio
FISH	Fluorescence *in situ* hybridization
IFAS	integrated fixed-film activated sludge
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
K_{DO}	Half-saturation constant of DO
K_La	Volumetric Oxygen Transfer Coefficient
MMCs	Mixed microbial cultures
$NADH_2$	Hydroxylamine reductase
NOB	Nitrite oxidizing bacteria
OLR	Organic loading rate
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHV	Polyhydroxyvalerate
SBR	Sequencing batch reactor
SND	Simultaneous nitrification and denitrification
SRT	Sludge retention time
TN	Total nitrogen
TSS	Total suspended solids
VFA	Volatile fatty acid
VSS	Volatile suspended solids
WWTP	Wastewater treatment plant

Nomenclature in chapter 4

$C_{\_acet}$/ $C_{\_prop}$/ $C_{\_but}$/ $C_{\_val}$	acetate, propionate, butyrate, and valerate concentrations (Cmmol/L)
$k_{\_j}$ ($k_{\_acet}$, $k_{\_prop}$, $k_{\_but}$ and $k_{\_val}$)	the fraction of the maximum uptake rate of each individual VFA on the maximum total VFA uptake rate (Cmol/Cmol)
X	active biomass concentration (Cmol/L)
$K_{\_i}$	inhibition factor of acetate and propionate when butyrate and valerate present (Cmmol/L)
δ	P/O ratio, efficiency of oxidative phosphorylation (molATP/mol$NADH_2$)
mATP/ $q_{\_mATP}$	maintenance coefficient on ATP (molATP/Cmol/h)
$K_{\_s\_VFA}$	half-saturation constant of VFAs (Cmmol/L)
$q_{\_decxl}$	decarboxylation rate (Cmol/Cmol/h)
$q_{\_VFA\_max}$	maximum total VFA uptake rate (Cmol/Cmol/h)

1

MOTIVATION AND THESIS OUTLINE

1.1 Motivation and objectives

Bio-based and biodegradable polymers nowadays play a very important role in promoting the circular economy of the plastic industry. Polyhydroxyalkanoates (PHAs), one group of the most promising biopolymer with a broad range of thermal and mechanical properties, are regarded as a potential replacement of traditional fossil based plastics.

Although PHA production by mixed microbial cultures has achieved high productivity with various polymer compositions, there is still a long way to go towards the full-scale commercial application. Several studies have been conducted aiming at optimizing the PHA production process by controlling different parameters, such as pH, temperature, organic loading rate, sludge retention time, etc. However, the aeration, which could affect directly the operational costs, was rarely discussed. Although the mechanism of PHA production from acetate, or a mixture of several volatile fatty acids (VFAs) has been discussed in literature, not all results observed experimentally can be described by the currently available metabolic models. On the other hand, in order to produce PHA as an effective method for carbon recovery from waste feedstocks, further studies are required for the integration of PHA production with the biological nutrient removal process in wastewater treatment plants.

The aims and scope of this thesis include:

- Studying the impact of dissolved oxygen (DO) levels on the mixed microbial culture selection and PHA accumulation stages.
- Developing a metabolic model to describe the substrate competition in PHA production by feeding multiple VFAs.
- Investigating the strategies of DO control for the integration of PHA production with the nitrogen removal process.

This work contributes towards the understanding of PHA production metabolism, optimization of the operational conditions for cost-effective PHA production processes, and provides suggestions for the implementation of PHA production in wastewater treatment processes.

1.2 Thesis outline

The thesis is composed of 7 chapters, describing the work conducted during this PhD project.

Chapter 1, the current chapter, presents the motivation and objectives, as well as the outline of this thesis.

Chapter 2 introduces the state of the art in biopolymer production by a brief literature review. This chapter focuses on the aspects of previous studies closely relating to the thesis work, such as the PHA production process and metabolism by mixed cultures, the impact of operational conditions, and the integration of PHA production with nutrient removal processes.

Chapter 3 describes the impact of DO level on the feast phase of culture selection and on the PHA accumulation stage. The selected microbial communities are characterized under both high and low feast DO levels, and the substrate competition among the VFAs (acetate, propionate, butyrate and valerate) is addressed.

This work has been published in an international peer reviewed scientific journal as:

Wang Xiaofei, Oehmen Adrian, Freitas Elisabete B, Carvalho Gilda, Reis Maria A M. 2017. "The Link of Feast-Phase Dissolved Oxygen (DO) with Substrate Competition and Microbial Selection in PHA Production." Water Research 112. Elsevier Ltd: 269–78. doi:http://dx.doi.org/10.1016/j.watres.2017.01.064.

Chapter 4 presents the metabolic model developed based on the experimental data from chapter 3, to describe the substrate competition, which has never been modeled before. This model provides deeper understanding on VFA uptake metabolism for PHA production, and contributes to the prediction of PHA composition and the optimization of the process efficiency.

This work has been submitted to an international peer reviewed scientific journal as:

Wang Xiaofei, Gilda Carvalho, Maria A M Reis, Adrian Oehmen. 2017. "Metabolic modeling of the substrate competition among multiple VFAs for PHA production by mixed microbial cultures". Journal of Biotechnology

In **chapter 5**, the study of DO impacts is further extended from the feast phase (in Chapter 3) to the entire cycle of culture selection, with an unlimited high DO level, and a strictly limiting low level. The impact of DO on microbial selection is characterized and the affinity of DO for the uptake of each VFA is quantified.

Wang Xiaofei, Carvalho Gilda, Oehmen Adrian, Reis Maria A M. "The impact of DO on PHA accumulating culture selection and the quantification of DO affinity on VFA uptake"

Chapter 6 discusses the influence of DO levels for PHA accumulation with concurrent nitrification processes in surplus municipal activated sludge. The DO affinities were determined for the two processes. Based on these results a process operation strategy is proposed through DO control to maintain PHA accumulation, while limiting the nitrification activities.

This work will be submitted to an international peer reviewed scientific journal as:

Wang Xiaofei, Bengtsson Simon, Oehmen Adrian, Carvalho Gilda, Werker Alan, Reis Maria A.M. "Application of dissolved oxygen level (DO) control for PHA accumulation with concurrent nitrification in surplus municipal activated sludge"

Chapter 7 concludes the main findings of this PhD project, and addresses the issues for future work.

2

STATE OF THE ART

2.1 Biopolymer production in the circular economy

The concept of "circular economy" was initiated in 1990´s by David W. Pearce and R. Kerry Turner, relating economy with environment, pollution, and natural resources, emphasizing their impacts on ethics and future generations (Pearce and Turner 1990). In contract to the linear economic model of the traditional industrial process, "take, make, and dispose", circular economy is a restorative and regenerative system, aiming at eliminating the waste for disposal, and keeping the utility and value of products all the time (Ellen MacArthur Foundation 2015).

As reported by the Ellen MacArthur Foundation, plastics and plastic packaging are important parts of global economy, however, the plastic material loses 95% of the value after the short first use, which causes USD 80-120 billion economic loss annually. Over 90% of the plastic productions nowadays still relies on the finite fossil feedstocks. At least 8 million tons of the wasted plastics leak into the ocean each year. The complex blend of chemical substances that emerged to plastics during manufacture would cause potential adverse effects on human health and the environment. (Ellen MacArthur Foundation, 2016)

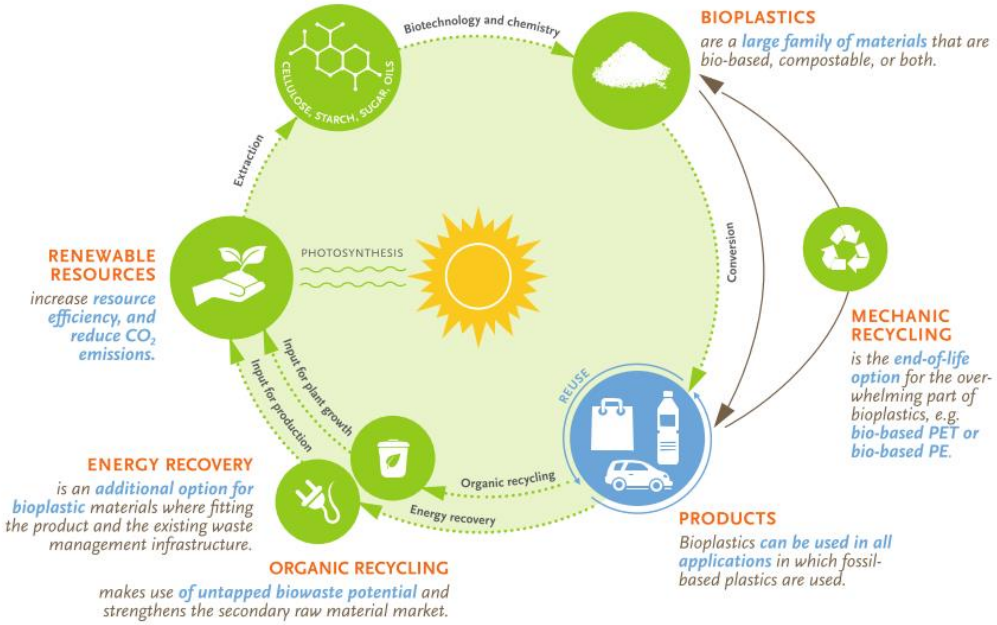

Figure 2-1 Bioplastics in the circular economy- closing the loop (European Bioplastics 2015)

Bioplastics fit perfectly in the loop of the circular economy of plastics industry (Figure 2-1), turning the source material from non-renewable petroleum based to recycled waste based, and providing the possibility of reuse and biodegradation of the end material (European Bioplastics 2015). Bioplastics can be classified as bio-based but not biodegradable plastics (eg. bio-polyamide (PA) and bio-polyethylene (PE)), biodegradable/compostable but not bio-based plastics (eg. poly (butylene adipate-co-terephthalate) (PBAT), Polybutylene succinate (PBS) and polycaprolactone (PCL)) and both bio-based and biodegradable plastics (eg. polyhydroxyalkanoates (PHA), polylactic acid (PLA) and cellophane)(van den Oever et al. 2017). The bio-based plastics which are made from organic feedstocks, such as corn, cellulose, sugar cane or cheese

whey, have received wide attention, with the potential to reach 10% of plastics production in the EU (European Bioplastics 2015). Compared to other waste management methods, like landfill and incineration, biodegradable/compostable plastics can help to collect biowaste, which contributes strongly to the organic recycling (European Bioplastics 2015).

2.2 Polyhydroxyalkanoates (PHA)

PHAs are bio-based and biodegradable polyesters that can be accumulated as intracellular energy and carbon reserves by various microorganisms (Lee 1996). As an important member of bioplastics, PHA is considered as a potential replacement of the conventional petroleum based plastics for packaging and other disposable or compostable items, due to the excellent thermal and mechanical properties (Reis et al. 2011; Chen 2009). Since some PHA monomers and oligomers exist naturally in human blood and tissue, the biocompatible properties of PHA provide the feasibility of the application in the medical field as drug delivery carriers or artificial implants (Chen 2009; Luef 2015).

Poly(3-hydroxybutyrate) (PHB), the first form of PHA, was discovered to be produced by *Bacillus megaterium* in 1926, by French microbiologist Maurice Lemoigne (Lemoigne 1926). Since then, over 90 different types of hydroxyalkanoate (HA) monomers have been identified (Steinbuchel and Valentin 1995). According to the length of the carbon chains, PHA can be classified into two groups: the short chain length (SCL) monomers with 3-5 carbon atoms, and the medium chain (MCL) monomers with 6-14 carbon atoms(Anderson and Dawes 1990). Generally, the PHA molecular weights range from 2×10^5 to 3×10^6 daltons, which means the linear PHA chain could be composed of up to 30 000 of the repeating units as shown in Figure 2-2 (Lee 1996).

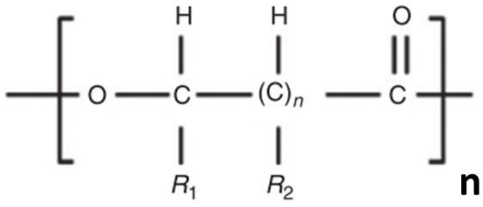

Figure 2-2 Polyhydroxyalkanoate (PHA) chemical structure. n: number of PHA units in the linear polyester structure; R1 and R2, variable hydrocarbon side chains (Reis et al. 2011)

The most widely applied and studied SCL PHAs are PHB and PHV (poly (3-hydroxyvalerate)). PHB has a compact helical structure, with the melting temperature close to 180 ºC and the glass transition temperature around 0 ºC (Anderson and Dawes 1990; Doi et al. 1990). PHB is relatively stiff due to its high crystallinity (60%-80%) (Anderson and Dawes 1990). By copolymerizing with other monomers like HV, the brittleness and poor melting stability of PHB could be significantly improved (Anderson and Dawes 1990; Lee 1996). The microstructure of the copolymer P(HB-HV) is generated statistically randomly due to the monomers distribution (Dai et al. 2008). Compared to the homopolymer PHB, the copolymer of 70mol-% HB and 30 mol-%

HV shows lower melting temperature (143 ºC), and increased flexibility with lower crystallinity (30%-40%) (Anderson and Dawes 1990; Lee 1996).

2.3 PHA production with pure cultures

The most studied and commercially applied PHA production method is using pure cultures of microorganisms. The wild type *Ralstonia eutropha*, formerly called *Alcaligenes eutrophus*, *Wautersia eutropha*,or *Cupriavidus necator*, is able to accumulate over 80% PHB fed by glucose, and over 75% PHBV when fed by a mixture of glucose and propionate (Chen 2009; Choi and Lee 1999). Another important industrial strain, the recombinant Escherichia coli, can produce over 75% P3HB4HB from glucose (Chen 2009; Choi and Lee 1999).

Notwithstanding the high PHA productivity that has been achieved in pure cultures, the high cost from the pure strains and substrates, and the sterilized operational conditions limited the large scale commercial production and application of PHAs as commodity bioplastics (Chanprateep 2010; Reis et al. 2011). As to the application in the medical field, the high requirements of biocompatible and biodegradable PHAs results in even higher prices (Luef 2015).

2.4 PHA production with mixed microbial cultures (MMCs)

Mixed microbial cultures have been proposed as a cost-effective method for PHA production, as compared to pure cultures (Chanprateep 2010; Reis et al. 2011). The activated sludge used for COD and nutrient removal in WWTPs contains enormous amounts of microorganisms with the capability of storing PHAs. These PHA storers can be selected as the mixed microbial cultures for efficient PHA production. Since the source of biomass is activated sludge, the cost for pure bacteria strains and the risk of culture degeneration are eliminated. In the meanwhile, the waste influent with high organic load could be applied as the carbon source for PHA production. Hence, the traditional waste management would potentially turn to a carbon recovery process producing biopolymers with added value. Open systems could be applied for the MMCs instead of the sterilized conditions when using pure culture, thus the energy cost for sterilization is avoided. Additionally, simple equipment and continuous processing are feasible in the PHA production by MMCs, which lead to the broad application of this process. (Reis et al. 2011; Albuquerque et al. 2010a; Johnson et al. 2009)

The commonly studied process for PHA production by MMCs consists of 3 stages (Takabatake et al. 2000), as sketched in Figure 2-3:

- Stage1: Anaerobic acidogenic fermentation.

Although many pure cultures were shown to be capable of converting carbohydrates directly to PHA (Reddy et al. 2003; Verlinden et al. 2007), in mixed cultures, it was found that carbohydrates such as glucose or starch followed preferably the pathways for glycogen synthesis (Dircks et al. 2001; Ö. Karahan et al. 2006). In comparison, VFAs were more suitable as substrates for PHA production (Carta et al. 2001; O. Karahan, Orhon, and Loosdrecht 2008). Anaerobic acidogenic fermentation is therefore conducted for converting the carbohydrates to

VFAs, controlling the operational conditions to avoid the production of methane subsequent to acidogenesis.

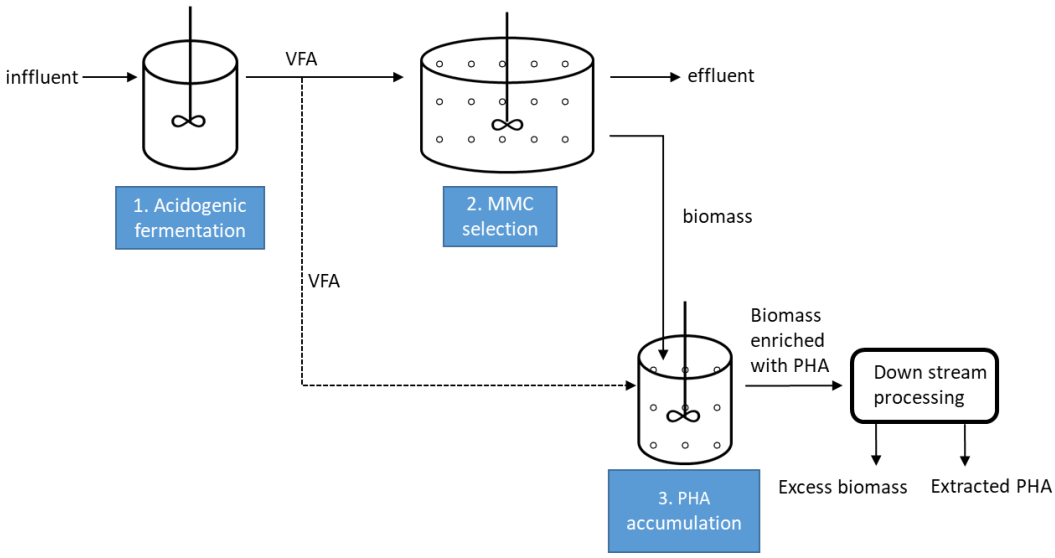

Figure 2-3 3-stage process for PHA production by MMCs

- Stage 2: MMC selection.

PHA accumulating bacteria are enriched by feeding the fermented substrates (VFAs) produced in stage 1. The feast and famine regime, also known as the aerobic dynamic feeding (ADF) is commonly applied for efficient MMC selection (Johnson et al. 2009; Albuquerque et al. 2010a; Jiang et al. 2012). In the short feast phase, when external substrates like VFAs are available, microorganisms take up the substrates for PHA storage. In the long famine phase, when external substrates are unavailable, the intracellular PHAs are utilized for cell growth and maintenance (Van Loosdrecht, Pot, and Heijnen 1997). The feast and famine regime offers a strong selective pressure for PHA accumulating bacteria. Other microorganisms that are not capable of storing PHAs are unable to grow in the famine phase due to the deficiency of carbon source and energy, thus they are washed out eventually. A typical feast and famine cycle is shown in Figure 2-4. However, in the feast phase, although VFAs are taken up primarily for PHA storage, cell growth would also occur when nutrients are present under the aerobic conditions. An advanced strategy of limiting the nutrients in the feast phase by only adding ammonia in the famine phase is applied in recent studies, which promotes the selection efficiency by enhancing the selective pressure (Oliveira et al. 2016).

The microbial population enriched is strongly dependent on the substrates and operational conditions (Albuquerque et al. 2013). The ability of PHA storage is a phylogenetic property of bacteria and over 300 species are known to synthesize PHA (Lee 1996). Commonly reported genera selected with high abundance by the feast and famine regime in MMCs include: *Thauera* (Davide Dionisi et al. 2006; Albuquerque et al. 2013; Queirós, Rossetti, and Serafim 2014), *Azoarcus* (Lemos et al. 2008; Carvalho et al. 2014), *Plasticicumulans* (Johnson et al. 2009; Jiang, Dimitry, et al. 2011), *Amaricoccus* (Lemos et al. 2008; Queirós, Rossetti, and Serafim

2014), *Paracoccus* (Albuquerque et al. 2013), *Meganema* (Majone et al. 2006; Coats, Watson, and Brinkman 2016), *Zoogloea* (Jiang, Marang, et al. 2011) *etc*.

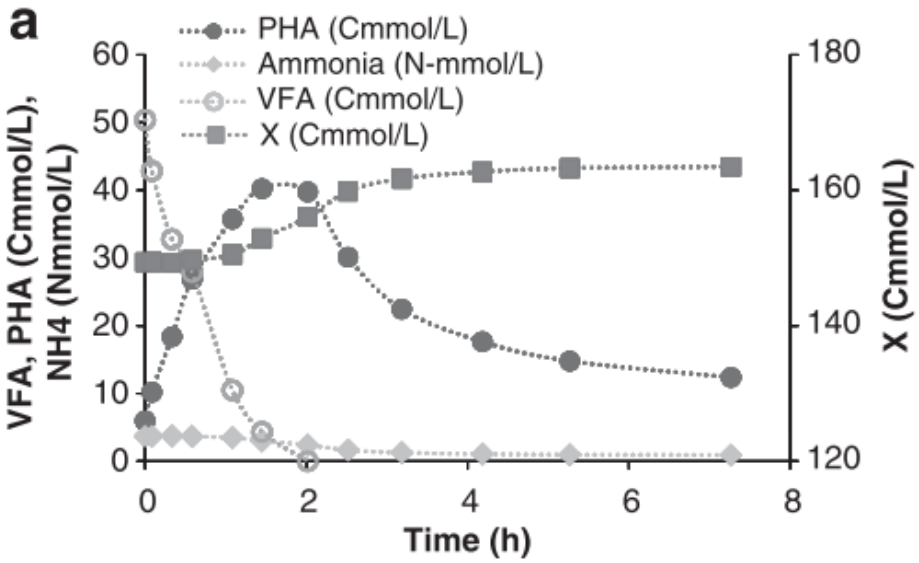

Figure 2-4 A typical feast and famine cycle in the culture selection stage (Albuquerque et al. 2013)

- Stage 3: PHA accumulation.

The maximum content of PHAs is accumulated in the microorganisms selected in stage 2, usually fed with the fermented substrates from stage 1, under aerobic feast conditions with additional carbon sources. The most commonly applied strategy for maximizing the PHA accumulation is pulse-wised feed-on-demand respirometric control (Serafim et al. 2004; Albuquerque et al. 2007; Jiang et al. 2012; Valentino et al. 2015). An increase of the respiration rate occurs when pulsing the feedstock, which corresponds to the decrease of the DO levels. When the substrate is depleted, the increase of DO level will trigger the next pulse of feedstock. The pulses are repeated till the DO level maintains at a certain level, thus, the maximum content of PHA in the biomass is achieved. The purpose for pulse-wised addition extends from the necessity to avoid substrate inhibition at high VFA concentrations.

Another method of supplying substrates is through pH control. A continuous feeding strategy was developed by Chen et al. (2015) based on adding VFAs with low pH. Under the optimal biomass loading rate 3.5–5.5 Cmol VFA/Cmol X/d, a high PHA content up to 70.4% and PHA production yield of 0.81Cmol PHA/Cmol VFA was achieved. The pH state was self-balanced when feeding the substrate at pH 5. This strategy showed higher PHA productivity than the pulse-wised method.

In most studies, nutrients were not provided in order to limit the cell growth (Dionisi et al. 2004; Albuquerque et al. 2010a; Johnson et al. 2009), however, a recent work showed that a certain

level of N and P addition (N/COD: 2-15 mg/g and P/COD: 0.5-3 mg/g) could promote the PHA productivity over the nutrient starving condition (Valentino et al. 2015).

2.5 PHA synthesis metabolism in mixed cultures

The metabolic pathways of PHA synthesis by MMCs from the commonly used VFAs, acetate, propionate, butyrate and valerate are illustrated in Figure 2-5. As explained by Reis et al. (2011) and Dias et al. (2008), each mole of VFA needs one mole ATP to be taken up into the cells via active transport. Acetate and propionate were converted to acetyl-CoA and propionyl-CoA, respectively. A part of the propionyl-CoA is converted to acetyl-CoA through decarboxylation and the remainder is reduced to propionyl-CoA*. When nutrients are unlimited, part of the acetyl-CoA enters the tricarboxylic acid (TCA) cycle for cell growth and maintenance. In order to accumulate as much PHA as possible, the nutrients are normally limited to drive more acetyl-CoA to acetyl-CoA* for PHA synthesis (Dionisi et al. 2004). HB is formed by the condensation of two acetyl-CoA* monomers, H2MV is generated by binding two propionyl-CoA* monomers, and HV (or 3-hydroxy-2methylbutyrate (H2MB), the isomer of HV) is produced by combining one acetyl-CoA* and one propionyl-CoA* monomer (Reis et al. 2011; Dias et al. 2008). Butyrate and valerate can be converted directly to HB and HV monomers, respectively (Reis et al. 2011).

The understanding of the PHA synthesis metabolism contributes to the development of metabolic models for describing and predicting the PHA production process (Jiang, Hebly, et al. 2011; Dias et al. 2005; Pardelha et al. 2014).

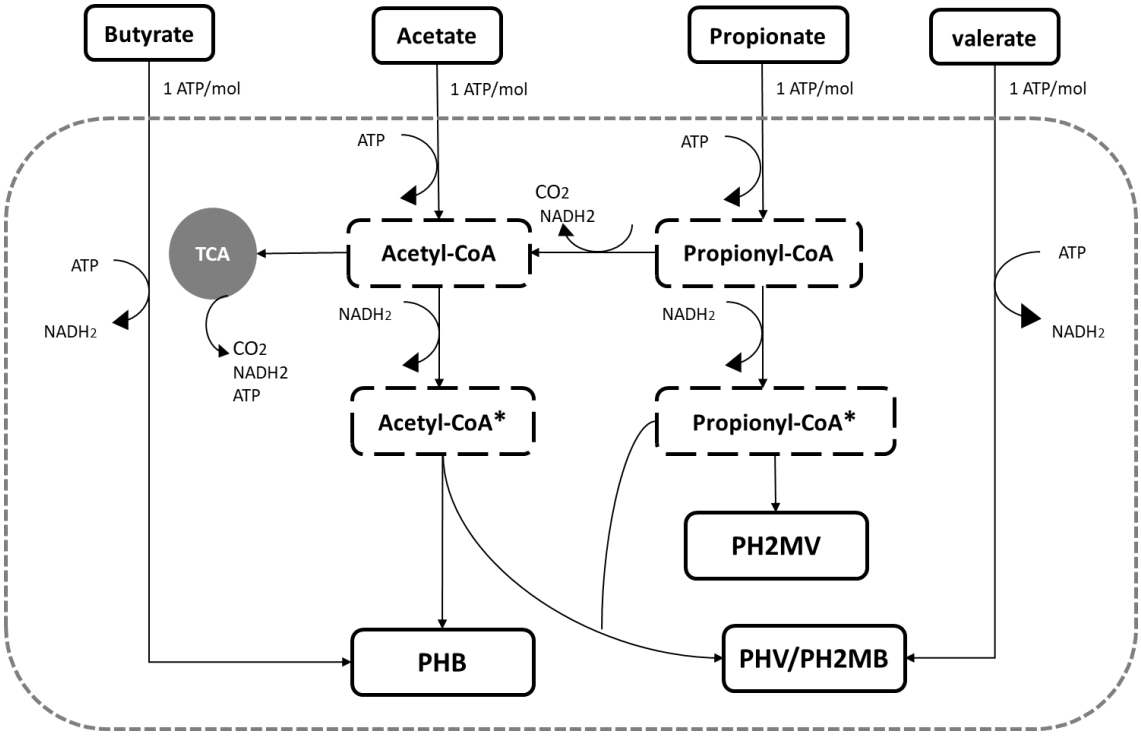

Figure 2-5 metabolic pathways of PHA synthesis from acetate, propionate, butyrate and valerate, adapted from (Reis et al. 2011; Pardelha et al. 2014)

2.6 The impact of operational condition on MMCs selection and PHA production process

The microbial composition and the PHA productivity are strongly dependent on the operational conditions for MMCs selection (step 2) and PHA accumulation (step 3). Thus, the operational conditions should be optimized for the successful culture enrichment with high PHA production yield and rate, and stable polymer composition. The impacts of several parameters were investigated by previous studies:

2.6.1 pH

The enrichment of PHA storers and PHA accumulating batches are normally operated under two conditions: without pH control (pH varying from 7 to 10) or under pH control at 7 to 8 by adding NaOH or HCl/H_2SO_4. Oehmen et al. (2014) investigated the impact of pH control on the MMCs selection fed by fermented molasses. Compared to the operation without pH control (maximum pH 9), controlling pH at 8 led to the double of the biomass concentration with no loss in the specific PHA storage efficiency, and the shift of the most abundant microbial population, from *Thauera* and *Paracoccus* to *Azoarcus*.

Serafim et al. (2004) compared the performance of PHB production in 3 batches, with pH controlled at 7, 8.3, and without control (pH 8-10), using the MMCs enriched under no pH control condition fed by acetate. It was found that under the condition with no pH control, the maximum PHB content and the polymer yield on the substrate were higher, as compared to the pH controlled conditions. The growth yield was not impacted by the pH.

Chua et al. (2003) pointed out that in the MMCs selection step, the PHA production capability and rates were not affected by the pH conditions in the range of 7-8. However, in the PHA accumulation step, pH higher than 8 was recommended, since the maximum pH content reduced to a very low level when controlled at 6 and 7, as compared to the pH conditions at 8 and 9.

A series of batch tests for PHA production were carried out by Dionisi et al. (2005) at a wider pH range of 4.5 -10.5, using the biomass enriched by feeding acetate, propionate and lactate at pH 7.5 without previous acclimation. The results showed that within the pH range 6.5-9.5, the biomass could maintain acceptable polymer production, while at lower pH (5.5 or 4.5) or higher pH (10.5), much lower PHA conversion yields, substrate consumption rates, PHA production rates were observed.

Additionally, pH was found to impact the polymer composition. According to Dionisi et al. (2005), the HV fraction in total PHA production increased from 10% to 30% by increasing the pH from 5.5 to 9.5. The same result was also reported by Villano et al. (2010). Higher HV content was achieved by the MMCs enriched in the SBR operated with higher pH. More HV was produced at pH 9.5 as compared to pH 7.5 and 8.5 in the accumulation batches, using the culture selected at pH 8.5. The reason of the increased HV content at higher pH could be the higher requirement of energy and biomass production via acetyl-CoA through the acetate metabolism,

which reduced the fraction of HB formation. Thus, the strategy of pH control could be used to achieve the P(HB/HV) copolymer with the designated HV fraction.

Overall, the literature studies suggest that pH control is not strictly necessary during PHA production, provided that it varies within the range of approximately 7 to 9.5, although it can impact the microbial community selection, which likely explains the contrasting results in certain cases.

2.6.2 Temperature

Room temperature (20-25 ºC) was the most preferable temperature setting in PHA production studies (Albuquerque et al. 2011; Majone et al. 2006; D. Dionisi et al. 2005). Krishna & Van Loosdrecht (1999) investigated the PHB production performance on acetate at different temperatures (15, 20, 25, 30 and 35 ºC) and found that the PHB formation rate was adversely impacted by the increase of temperature due to the higher requirement of maintenance and endogenous processes. A more recent study showed that temperature had a major impact on the microbial composition in the MMC selection step but not on the PHA production performance (Jiang, Marang, et al. 2011). The dominant population selected at 20 ºC and 30 ºC was *Zoogloea* and *Plasticicumulans*, respectively. Compared to *Zoogloea*, *Plasticicumulans* was more sensitive to temperature changes.

2.6.3 Sludge retention time (SRT)

SRT theoretically determines the life-time of the microbial communities, which can impact both the microbial selection and the process performance of PHA production. It was reported that the propionate-favored bacteria *Amaricoccus* was selected at longer SRT (10 days) with the high percentage at 61.4±1.9% of the total bacteria, but almost completely removed at shorter SRT (1 day) (Lemos et al. 2008). They also found lower yield and PHB production rate at SRT of 1 day as compared to SRT of 10 days when using acetate as the carbon source. However, contradictory results were obtained by Chua et al. (2003). Higher PHA production capacity was achieved with SRT of 3 days as compared to 10 days in their study. Johnson et al. (2009) achieved successful *Plasticicumulans* dominating culture selection with high PHA accumulating potential (up to 89 wt %) at 1 day SRT. On one hand, shorter SRT means faster growth rate, which may require a higher fraction of the substrate being consumed for growth instead of PHA storage (Beun et al. 2000). On the other hand, the shorter SRT may promote the selection of the microorganisms with higher PHA storage capacity, while the culture selected with longer SRT with higher dry weight normally contains higher fraction of inert cells (Chua et al. 2003). It was suggested by a metabolic model to operate the SBR at SRT above 2 days for stable PHA accumulating culture selection with constant PHA production yield around 0.6 Cmol/Cmol (Beun et al. 2002).

2.6.4 Organic loading rate (OLR)

The organic loading rate plays an important role together with the SRT in the establishment of effective and stable feast and famine conditions for PHA accumulating culture enrichment. In

principle, higher OLR and shorter SRT are desired for high PHA productivity and growth rates. Several studies have been conducted to investigate the optimization of OLR in the culture selection step. Dionisi et al. (2006) fed a SBR with a mixture of acetic, lactic and propionic acids at different OLRs (8.5-31.25 gCOD/L/d) and obtained high biomass productivity and PHA storage at an intermediate OLR of 20 gCOD/L/d. Another study by Albuquerque et al. (2010b) applied the fermented sugar molasses as the substrate for the PHA accumulating culture selection at the OLR of 60, 90 and 120 CmmolVFA/L/d, respectively. It was found that at the lowest OLR 60 CmmolVFA/L/d, the process kinetics were limited on substrate availability; while at OLR 120 CmmolVFA/L/d, the growth limitation was observed due to micronutrient deficiency, which led to a variation of feast and famine ratio. In their study, the optimal OLR was at 90 CmmolVFA/L/d, whereby a culture of 88% PHA-storing bacteria was enriched, with the maximum PHA content up to 74.6%. The impact of OLR on the PHA production process fed by fermented olive oil mill wastewater was also investigated (Campanari et al. 2014). 4.7 gCOD/L/d was reported as the optimal OLR in the range of 2.4 – 8.4 gCOD/L/d, with the highest PHA storage rate and yield. Overall, the optimal OLR is strongly dependent on the composition of the substrate, and also relates to other operational conditions.

2.6.5 Dissolved oxygen (DO)

Oxygen input is more directly related to process cost, as compared to other operational conditions, since a large amount of energy is required for aeration in the aerobic process of PHA production. It was found that in the SBRs for culture selection, low DO condition (k_La 6 to 16h^{-1}, 0 mg/L DO) promoted the PHB production with higher yield, while under high DO condition (k_La 30, 51 h^{-1}, DO >0.9 mg/L), a higher fraction of the substrate (acetate) was used for biomass growth (Third, Newland, and Cord-Ruwisch 2003). The reason for this result is that the PHA production process required less energy than cell growth. When the ATP availability was limited with low DO concentrations, the PHA production process was favored. In a recent study, Coats et al. (2016) applied aeration strategies with a different range of k_La (4, 8, 12, 20 h^{-1}) in the MMC selection step fed by VFA-rich fermented dairy manure. No statistical difference in the PHA accumulation rates was observed. Although the DO concentrations varied in the famine phase due to the different k_La applied, the DO patterns were similar under all conditions in the feast phase, where the main activities of substrate uptake and PHA production occurred. Vjayan & Vadivelu (2017) concluded that the reduction of air flow (from 1 vvm to 0.5, 0.25 and 0 vvm) in the famine phase in aerobic granules had no significant impact on the COD removal and PHA content.

Pratt et al. (2012) studied the DO impact on the PHA accumulation step, using a culture enriched under unlimited DO conditions fed by fermented dairy waste. The maximum PHA content reached the same level around 35% of the cell dry weight, at both DO concentrations of 0.2 mg/L and 5.5 mg/L. However, it took three times longer to reach the same maximum PHA content at the low DO level, as compared to the high DO level.

More work is needed to explore the impact of DO concentrations to the PHA production process. It seems that an optimized low DO level could be applied as the controlling strategy to promote PHA productivity while inhibiting the biomass growth, and at the same time, saving the energy costs.

2.7 Integrating PHA production and nutrient removal processes

Recent studies showed that PHA-storing biomass was able to be enriched in the wastewater treatment process aiming at removing COD and nutrients in the WWTP (Oehmen et al. 2007; Anterrieu et al. 2014; Morgan-sagastume et al. 2015; Bengtsson, 2017b). Different microbial communities following their respective metabolism for PHA production were found in the biological nutrient removal processes.

In the enhanced biological phosphorus removal (EBPR) process under anaerobic/aerobic conditions, two groups of microorganisms, polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs), are able to store PHAs intracellularly (Oehmen et al. 2007). Under anaerobic conditions, PAOs accumulate PHAs by taking up extracellular carbon like VFAs, along with the P release and glycogen glycolysis. In the following aerobic conditions, PAOs use the stored PHAs as carbon source for biomass synthesis, glycogen replenishment and converting P to polyphosphate. Since GAOs have no contribution to P removal, they acquire ATP from glycogen for anaerobic PHAs formation, then use the PHAs aerobically for cell growth and glycogen replenishment (Oehmen et al. 2007). Even though PHA was commonly recognized as an intermediate in phosphate removal, the PHA accumulation potential (PAP) of these microbial communities could not be neglected. Bengtsson et al. (2008) enriched a culture dominated by GAOs by alternating anaerobic/aerobic conditions, fed with fermented paper mill wastewater. They achieved a high PHA content up to 42% cell dry weight, by aerobic/anaerobic PHA accumulating strategy.

In the conventional A/O (anoxic-aerobic) process for N removal in WWTP, PHA accumulation is commonly observed in the anoxic tank for denitrification. The PHA-accumulating communities, some of them also performing as denitrifiers, can convert readily biodegradable COD (RB COD) to PHAs, then use the PHA stored for cell growth and maintenance in the aerobic tank. Hence, microorganisms with high PHA accumulation potential are selected under the anoxic-feast and aerobic-famine conditions. (Anterrieu et al. 2014; Bengtsson et al. 2017b).

In the A2O (anaerobic-anoxic-aerobic) process integrating both N and P removal, PHA is firstly accumulated in the anaerobic tank. It is worth noticing that PAOs and GAOs for PHA production are only able to consume specific organic substrates like VFAs. The remaining RBCOD is consumed in the following anoxic process. Compared to the A2O process, the A/O process is preferable as the feast-famine selector, since the A/O process allows the uptake of a broader range of RBCOD sources in the anoxic tank as the first step, which places a strong selective pressure on PHA-accumulating communities under readily established anoxic-feast conditions (Bengtsson et al. 2017b).

The high PHA accumulation potential of non-selected activated sludge (Bengtsson et al. 2017b) and the possibility to use municipal wastewater containing RBCOD directly as feedstock (Morgan-sagastume et al. 2015) provide the chance to integrate PHA production with traditional wastewater treatment for nutrient removal. Several studies have been conducted to investigate the strategy and mechanism of the integration of the two processes. Anterrieu et al. (2014) integrated the nutrient removal process with PHA production using the condensate and wash-water from a sugar factory. They performed 2 lab scale SBRs, one with conventional activated sludge and the other with hybrid biofilm activated sludge. The biomass (with PAP >60% g-PHA/g-VSS) of both processes accumulate PHAs under anoxic-feast and consume PHAs under aerobic-famine, while removing nutrients. Bengtsson et al. (2017a) extended the biological municipal wastewater treatment process for carbon and nitrogen removal while producing PHAs to pilot scale. The process, using integrated fixed-film activated sludge with biofilm carrier media, was composed by 3 steps: pre-denitrification, nitrification and post-nitrification. The first two steps simulated the anoxic-feast and aerobic-famine for PHA production and consumption, and the third step aimed at removing the residual nitrate. Satisfactory COD and nitrogen removal (83% and 80%, respectively) was achieved, while high PAP up to 49% g-PHA/g-VSS was obtained by the biomass.

An alternative strategy, aerobic-feast and anoxic-famine for PHA production integrating nutrient removal, was also investigated. Frison et al. (2015) selected PHA storing bacteria integrating the side stream treatment of nitrogen removal (via nitrite) from sewage sludge reject water. Under aerobic conditions, ammonium was oxidized to nitrite through nitritation, while VFAs were converted to PHAs; under anoxic conditions, N was removed via denitritation and PHAs were consumed internally as carbon sources. This study was conducted in two configurations, configuration 1 with all processes occurred in a single SBR, and configuration 2 with separated SBRs for nitritation and denitritation, respectively. PHA accumulating biomass was successfully selected in both configurations, while higher nitrogen removal efficiency with PAP around 40% g-PHA/g-VSS was observed in configuration 2. Basset et al. (2016) applied a similar methodology to develop a novel scheme for simultaneous N removal and PHA recovery in the main wastewater treatment line. The process consisted of a SBR for nitritation/denitritation and PHA storing biomass selection, a batch reactor for PHA accumulation, and a fermentation reactor for producing VFAs from sewage sludge and municipal solid waste.

Notwithstanding the fact that both strategies, anoxic-feast/aerobic-famine and aerobic-feast/anoxic famine, integrated successfully the PHA production and nutrient removal processes, the choice of the strategy depended largely on the configuration of the wastewater treatment plant as well as the composition of the influent waste water. Compared to the anoxic-feast, the aerobic-feast required a higher concentration of RBCOD or VFAs in the substrate, since under aerobic conditions, the substrates were initially consumed for both PHA storage and other aerobic activities like cell growth.

References

Albuquerque, M. G E, M. Eiroa, C. Torres, B. R. Nunes, and M. a M Reis. 2007. "Strategies for the Development of a Side Stream Process for Polyhydroxyalkanoate (PHA) Production from Sugar Cane Molasses." *Journal of Biotechnology* 130 (4): 411–21. doi:10.1016/j.jbiotec.2007.05.011.

Albuquerque, M.G.E., S. Concas, S. Bengtsson, and M.A.M. Reis. 2010a. "Mixed Culture Polyhydroxyalkanoates Production from Sugar Molasses: The Use of a 2-Stage CSTR System for Culture Selection." Bioresource Technology 101 (18). Elsevier Ltd: 7112–22. doi:10.1016/j.biortech.2010.04.019.

Albuquerque, M G E, V Martino, E Pollet, L Avérous, and M A M Reis. 2011. "Mixed Culture Polyhydroxyalkanoate (PHA) Production from Volatile Fatty Acid (VFA)-Rich Streams: Effect of Substrate Composition and Feeding Regime on PHA Productivity, Composition and Properties." *Journal of Biotechnology* 151 (1): 66–76. doi:10.1016/j.jbiotec.2010.10.070.

Albuquerque, M G E, C A V Torres, and M A M Reis. 2010b. "Polyhydroxyalkanoate (PHA) Production by a Mixed Microbial Culture Using Sugar Molasses: Effect of the Influent Substrate Concentration on Culture Selection." Water Research 44 (11): 3419–33. doi:http://dx.doi.org/10.1016/j.watres.2010.03.021.

Albuquerque, Maria G E, Gilda Carvalho, Caroline Kragelund, Ana F Silva, Maria T Barreto Crespo, Maria A M Reis, and Per H Nielsen. 2013. "Link between Microbial Composition and Carbon Substrate-Uptake Preferences in a PHA-Storing Community." *The ISME Journal* (2013) 7,1 (1751–7362/13).

Anderson, Alistair J, and Edwin A Dawes. 1990. "Occurrence, Metabolism, Metabolic Role, and Industrial Uses of Bacterial Polyhydroxyalkanoates." *Microbiological Reviews* 54 (4): 450–72. doi:0146-0749/90/040450-23$02.00/0.

Anterrieu, Simon, Luca Quadri, Bert Geurkink, Inez Dinkla, Simon Bengtsson, Monica Arcos-hernandez, Tomas Alexandersson, et al. 2014. "Integration of Biopolymer Production with Process Water Treatment at a Sugar Factory." *New BIOTECHNOLOGY* 31 (4). Elsevier B.V.: 308–23. doi:10.1016/j.nbt.2013.11.008.

Basset, N., E. Katsou, N. Frison, S. Malamis, J. Dosta, and F. Fatone. 2016. "Integrating the Selection of PHA Storing Biomass and Nitrogen Removal via Nitrite in the Main Wastewater Treatment Line." *Bioresource Technology* 200. Elsevier Ltd: 820–29. doi:10.1016/j.biortech.2015.10.063.

Bengtsson, Simon, Anton Karlsson, Tomas Alexandersson, Luca Quadri, Markus Hjort, Peter Johansson, Fernando Morgan-Sagastume, et al. 2017a. "A Process for Polyhydroxyalkanoate (PHA) Production from Municipal Wastewater Treatment with Biological Carbon and Nitrogen Removal Demonstrated at Pilot-Scale." New Biotechnology 35 (November). Elsevier B.V.: 42–53. doi:10.1016/j.nbt.2016.11.005.

Bengtsson, Simon, Alan Werker, Cindy Visser, and Leon Korving. 2017b. "PHARIO Stepping Stone to a Sustainable Value Chain for PHA Bioplastic Using Municipal Activated Sludge."

Bengtsson, Simon, Alan Werker, and Thomas Welander. 2008. "Production of Polyhydroxyalkanoates by Glycogen Accumulating Organisms Treating a Paper Mill Wastewater." *Water Science & Technology*, no. 2: 323–30. doi:10.2166/wst.2008.381.

Beun, J. J., K. Dircks, M. C M Van Loosdrecht, and J. J. Heijnen. 2002. "Poly-B-Hydroxybutyrate

Metabolism in Dynamically Fed Mixed Microbial Cultures." *Water Research* 36 (5): 1167–80. doi:10.1016/S0043-1354(01)00317-7.

Beun, J. J., F. Paletta, M. C M Van Loosdrecht, and J. J. Heijnen. 2000. "Stoichiometry and Kinetics of Poly-β-Hydroxybutyrate Metabolism in Aerobic, Slow Growing, Activated Sludge Cultures." *Biotechnology and Bioengineering* 67 (4): 379–89. doi:10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2.

Campanari, Sabrina, Francisca a E Silva, Lorenzo Bertin, Marianna Villano, and Mauro Majone. 2014. "Effect of the Organic Loading Rate on the Production of Polyhydroxyalkanoates in a Multi-Stage Process Aimed at the Valorization of Olive Oil Mill Wastewater." *International Journal of Biological Macromolecules* 71 (November). Elsevier B.V.: 34–41. doi:10.1016/j.ijbiomac.2014.06.006.

Carta, F, J.J Beun, M.C.M van Loosdrecht, and J.J Heijnen. 2001. "Simultaneous Storage and Degradation of Phb and Glycogen in Activated Sludge Cultures." *Water Research* 35 (11): 2693–2701. doi:10.1016/S0043-1354(00)00563-7.

Carvalho, Gilda, Adrian Oehmen, Maria G E Albuquerque, and Maria a M Reis. 2014. "The Relationship between Mixed Microbial Culture Composition and PHA Production Performance from Fermented Molasses." *New Biotechnology* 31 (4). Elsevier B.V.: 257–63. doi:10.1016/j.nbt.2013.08.010.

Chanprateep, Suchada. 2010. "Current Trends in Biodegradable Polyhydroxyalkanoates." *Journal of Bioscience and Bioengineering* 110 (6). The Society for Biotechnology, Japan: 621–32. doi:10.1016/j.jbiosc.2010.07.014.

Chen, Guo-Qiang. 2009. "A Microbial Polyhydroxyalkanoates (PHA) Based Bio- and Materials Industry." *Chemical Society Reviews* 38 (8): 2434. doi:10.1039/b812677c.

Chen, Zhiqiang, Long Huang, Qinxue Wen, and Zirui Guo. 2015. "Efficient Polyhydroxyalkanoate (PHA) Accumulation by a New Continuous Feeding Mode in Three-Stage Mixed Microbial Culture (MMC) PHA Production Process." *Journal of Biotechnology* 209 (October). Elsevier B.V.: 68–75. doi:10.1016/j.jbiotec.2015.06.382.

Choi, J., and S. Y. Lee. 1999. "Factors Affecting the Economics of Polyhydroxyalkanoate Production by Bacterial Fermentation." *Applied Microbiology and Biotechnology* 51 (1): 13–21. doi:10.1007/s002530051357.

Chua, Adeline S.M., Hiroo Takabatake, Hiroyasu Satoh, and Takashi Mino. 2003. "Production of Polyhydroxyalkanoates (PHA) by Activated Sludge Treating Municipal Wastewater: Effect of pH, Sludge Retention Time (SRT), and Acetate Concentration in Influent." *Water Research* 37 (15): 3602–11. doi:10.1016/S0043-1354(03)00252-5.

Coats, Erik R, Benjamin S Watson, and Cynthia K Brinkman. 2016. "Polyhydroxyalkanoate Synthesis by Mixed Microbial Consortia Cultured on Fermented Dairy Manure : Effect of Aeration on Process Rates / Yields and the Associated Microbial Ecology." *Water Research* 106. Elsevier Ltd: 26–40. doi:10.1016/j.watres.2016.09.039.

Dai, Yu, Lynette Lambert, Zhiguo Yuan, and Jurg Keller. 2008. "Microstructure of Copolymers of Polyhydroxyalkanoates Produced by Glycogen Accumulating Organisms with Acetate as the Sole Carbon Source." *Process Biochemistry* 43 (9): 968–77. doi:10.1016/j.procbio.2008.04.023.

Dias, João M.L., Luísa S. Serafim, Paulo C. Lemos, Maria a.M. Reis, and Rui Oliveira. 2005. "Mathematical Modelling of a Mixed Culture Cultivation Process for the Production of

Polyhydroxybutyrate." *Biotechnology and Bioengineering* 92 (2): 209–22. doi:10.1002/bit.20598.

Dias, João M L, Adrian Oehmen, Luísa S Serafim, Paulo C Lemos, Maria A M Reis, and Rui Oliveira. 2008. "Metabolic Modelling of Polyhydroxyalkanoate Copolymers Production by Mixed Microbial Cultures." *BMC Systems Biology* 2 (1): 59. doi:10.1186/1752-0509-2-59.

Dionisi, D., G. Carucci, M. Petrangeli Papini, C. Riccardi, M. Majone, and F. Carrasco. 2005. "Olive Oil Mill Effluents as a Feedstock for Production of Biodegradable Polymers." *Water Research* 39 (10): 2076–84. doi:10.1016/j.watres.2005.03.011.

Dionisi, D, V Renzi, M Majone, M Beccari, and R Ramadori. 2004. "Storage of Substrate Mixtures by Activated Sludges under Dynamic Conditions in Anoxic or Aerobic Environments" 38: 2196–2206. doi:10.1016/j.watres.2004.01.018.

Dionisi, Davide, Mario Beccari, Simona D. Gregorio, Mauro Majone, Marco Petrangeli Papini, and Giovanni Vallini. 2005. "Storage of Biodegradable Polymers by an Enriched Microbial Community in a Sequencing Batch Reactor Operated at High Organic Load Rate." *Journal of Chemical Technology and Biotechnology* 80 (December 2004): 1306–18. doi:10.1002/jctb.1331.

Dionisi, Davide, Mauro Majone, Giovanni Vallini, Simona Di Gregorio, and Mario Beccari. 2006. "Effect of the Applied Organic Load Rate on Biodegradable Polymer Production by Mixed Microbial Cultures in a Sequencing Batch Reactor." *Biotechnology and Bioengineering* 93 (1): 76–88. doi:10.1002/bit.20683.

Dircks, K., J. J. Beun, M. van Loosdrecht, J. J. Heijnen, and M. Henze. 2001. "Glycogen Metabolism in Aerobic Mixed Cultures." *Biotechnology and Bioengineering* 73 (2): 85–94. doi:10.1002/bit.1040.

Doi, Yoshiharu, Youko Kanesawa, Masao Kunioka, and Terumi Saito. 1990. "Biodegradation of Microbial Copolyesters: Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) and Poly(3-Hydroxybutyrate-Co-4-Hydroxybutyrate)." *Macromolecules* 23 (1): 26–31. doi:10.1021/ma00203a006.

Ellen MacArthur Foundation. 2015. "Towards a Circular Economy: Business Rationale for an Accelerated Transition," 20. doi:2012-04-03.

Ellen MacArthur Foundation the World Economic Forum and McKinsey & Company. 2016. "The New Plastics Economy: Rethinking the Future of Plastics." *Ellen MacArthur Foundation the World Economic Forum and McKinsey & Company*. doi:10.1103/Physrevb.74.035409.

European Bioplastics. 2015. "Bioplastics and the Circular Economy." http://docs.european-bioplastics.org/2016/publications/fs/EUBP_fs_end-of-life.pdf.

Frison, Nicola, Evina Katsou, Simos Malamis, Adrian Oehmen, and Francesco Fatone. 2015. "Development of a Novel Process Integrating the Treatment of Sludge Reject Water and the Production of Polyhydroxyalkanoates (PHAs)." doi:10.1021/acs.est.5b01776.

Jiang, Yang, Yu Dimitry, Robbert Kleerebezem, Gerard Muyzer, and Mark van Loosdrecht. 2011. "Plasticicumulans Acidivorans Gen. Nov., Sp. Nov., a Polyhydroxyalkanoate-Accumulating Gammaproteobacterium from a Sequencing-Batch Bioreactor." *International Journal of Systematic and Evolutionary Microbiology* 61 (9): 2314–19. doi:10.1099/ijs.0.021410-0.

Jiang, Yang, Marit Hebly, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2011. "Metabolic Modeling of Mixed Substrate Uptake for Polyhydroxyalkanoate (PHA)

Production." *Water Research* 45 (3): 1309–21. doi:10.1016/j.watres.2010.10.009.

Jiang, Yang, Leonie Marang, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2011. "Effect of Temperature and Cycle Length on Microbial Competition in PHB-Producing Sequencing Batch Reactor." *The ISME Journal* 5 (5). Nature Publishing Group: 896–907. doi:10.1038/ismej.2010.174.

Jiang, Yang, Leonie Marang, Jelmer Tamis, Mark C M van Loosdrecht, Henk Dijkman, and Robbert Kleerebezem. 2012. "Waste to Resource: Converting Paper Mill Wastewater to Bioplastic." *Water Research* 46 (17). Elsevier Ltd: 5517–30. doi:10.1016/j.watres.2012.07.028.

Johnson, Katja, Yang Jiang, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2009. "Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity." *Biomacromolecules* 10 (4). American Chemical Society: 670–76. doi:10.1021/bm8013796.

Karahan, Özlem, António Martins, Derin Orhon, and Mark C.M. van Loosdrecht. 2006. "Experimental Evaluation of Starch Utilization Mechanism by Activated Sludge." *Biotechnology and Bioengineering* 93 (5): 964–70. doi:10.1002/bit.20795.

Karahan, Ozlem, Derin Orhon, and Mark C. M. van Loosdrecht. 2008. "Simultaneous Storage and Utilization of Polyhydroxyalkanoates and Glycogen under Aerobic Conditions." *Water Science & Technology* 58 (4): 945. doi:10.2166/wst.2008.403.

Krishna, Chundakkadu, and Mark C.M. Van Loosdrecht. 1999. "Effect of Temperature on Storage Polymers and Settleability of Activated Sludge." *Water Research* 33 (10): 2374–82. doi:10.1016/S0043-1354(98)00445-X.

Lee, Sang Yup. 1996. "Bacterial Polyb Ydroxyalkanoates." *Biotechnology and Bioengineering* 49: 1–14. doi:10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P.

Lemoigne, M. 1926. "The Production of Acid and Beta-Oxybutyric Lactides by Bacillus Mesesentericus Vulgatus." *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* 94. http://findit.dtu.dk/en/catalog/157965026.

Lemos, Paulo C, Caterina Levantesi, Luisa S Serafim, Simona Rossetti, Maria a M Reis, and Valter Tandoi. 2008. "Microbial Characterisation of Polyhydroxyalkanoates Storing Populations Selected under Different Operating Conditions Using a Cell-Sorting RT-PCR Approach." *Applied Microbiology and Biotechnology* 78 (2): 351–60. doi:10.1007/s00253-007-1301-5.

Luef, K. P. 2015. "Poly(hydroxy Alkanoate)s in Medical Applications." *Chemical and Biochemical Engineering Quarterly* 29 (2): 287–97. doi:10.15255/CABEQ.2014.2261.

Majone, Mauro, Mario Beccari, S. Di Gregorio, Dadive Dionisi, and Giovanni Vallini. 2006. "Enrichment of Activated Sludge in a Sequencing Batch Reactor for Polyhydroxyalkanoate Production." *Water Science and Technology* 54 (1): 119–28. doi:10.2166/wst.2006.379.

Morgan-sagastume, F, M Hjort, D Cirne, F Gérardin, S Lacroix, G Gaval, and L Karabegovic. 2015. "Bioresource Technology Integrated Production of Polyhydroxyalkanoates (PHAs) with Municipal Wastewater and Sludge Treatment at Pilot Scale." *BIORESOURCE TECHNOLOGY* 181. Elsevier Ltd: 78–89. doi:10.1016/j.biortech.2015.01.046.

Oehmen, Adrian, Paulo C Lemos, Gilda Carvalho, Zhiguo Yuan, Linda L Blackall, and Maria A M Reis. 2007. "Advances in Enhanced Biological Phosphorus Removal : From Micro to Macro Scale" 41: 2271–2300. doi:10.1016/j.watres.2007.02.030.

Oehmen, Adrian, Fátima V. Pinto, Vera Silva, Maria G.E. Albuquerque, and Maria A.M. Reis. 2014. “The Impact of pH Control on the Volumetric Productivity of Mixed Culture PHA Production from Fermented Molasses.” *Engineering in Life Sciences* 14 (2): 143–52. doi:10.1002/elsc.201200220.

Oliveira, Catarina S.S., Carlos E. Silva, Gilda Carvalho, and Maria A. Reis. 2016. “Strategies for Efficiently Selecting PHA Producing Mixed Microbial Cultures Using Complex Feedstocks: Feast and Famine Regime and Uncoupled Carbon and Nitrogen Availabilities.” *New Biotechnology*. Elsevier B.V. doi:10.1016/j.nbt.2016.10.008.

Pardelha, Filipa, Maria G E Albuquerque, Maria a M Reis, Rui Oliveira, and João M L Dias. 2014. “Dynamic Metabolic Modelling of Volatile Fatty Acids Conversion to Polyhydroxyalkanoates by a Mixed Microbial Culture.” *New Biotechnology* 31 (4): 335–44. doi:10.1016/j.nbt.2013.06.008.

Pearce, D W, and R K Turner. 1990. *Economics of Natural Resources and the Environment*. *Harvester Wheatsheaf, London*. Harvester Wheatsheaf, London.

Pratt, S., a. Werker, F. Morgan-Sagastume, and P. Lant. 2012. “Microaerophilic Conditions Support Elevated Mixed Culture Polyhydroxyalkanoate (PHA) Yields, but Result in Decreased PHA Production Rates.” *Water Science & Technology* 65 (2): 243. doi:10.2166/wst.2012.086.

Queirós, Diogo, Simona Rossetti, and Luísa S Serafim. 2014. “PHA Production by Mixed Cultures: A Way to Valorize Wastes from Pulp Industry.” *Bioresource Technology*, no. 0. doi:http://dx.doi.org/10.1016/j.biortech.2014.01.099.

Reddy, C.S.K, R Ghai, Rashmi, and V.C Kalia. 2003. “Polyhydroxyalkanoates: An Overview.” *Bioresource Technology* 87 (2): 137–46. doi:10.1016/S0960-8524(02)00212-2.

Reis, M, M Albuquerque, F C T Universidade, and Nova De Lisboa. 2011. *Comprehensive Biotechnology*. *Comprehensive Biotechnology*. Second Edi. Vol. 1. Elsevier. doi:10.1016/B978-0-08-088504-9.00464-5.

Serafim, Luísa S., Paulo C. Lemos, Rui Oliveira, and Maria a M Reis. 2004. “Optimization of Polyhydroxybutyrate Production by Mixed Cultures Submitted to Aerobic Dynamic Feeding Conditions.” *Biotechnology and Bioengineering* 87 (2): 145–60. doi:10.1002/bit.20085.

Steinbuchel, Alxander, and Henry E Valentin. 1995. “Diversity of Bacterial Polyhydroxyalkanoic Acids.” *FEMS Microbiology Letters* 128: 219–28.

Takabatake, H., H. Satoh, T. Mino, and T. Matsuo. 2000. “Recovery of Biodegradable Plastics from Activated Sludge Process.” *Water Science and Technology* 42 (3–4): 351–56.

Third, Katie a., Mark Newland, and Ralf Cord-Ruwisch. 2003. “The Effect of Dissolved Oxygen on PHB Accumulation in Activated Sludge Cultures.” *Biotechnology and Bioengineering* 82 (2): 238–50. doi:10.1002/bit.10564.

Valentino, Francesco, Lamija Karabegovic, Mauro Majone, Fernando Morgan-sagastume, and Alan Werker. 2015. “Polyhydroxyalkanoate (PHA) Storage within a Mixed-Culture Biomass with Simultaneous Growth as a Function of Accumulation Substrate Nitrogen and Phosphorus Levels.” *Water Research* 77. Elsevier Ltd: 49–63. doi:10.1016/j.watres.2015.03.016.

van den Oever, Martien, Karin Molenveld, Maarten van der Zee, and Harriëtte Bos. 2017. “Bio-Based and Biodegradable Plastics – Facts and Figures.”

Van Loosdrecht, M. C M, M. a. Pot, and J. J. Heijnen. 1997. “Importance of Bacterial Storage Polymers in Bioprocesses.” *Water Science and Technology* 35 (1): 41–47. doi:10.1016/S0273-1223(96)00877-3.

Verlinden, R.A.J., D.J. Hill, M.A. Kenward, C.D. Williams, and I. Radecka. 2007. “Bacterial Synthesis of Biodegradable Polyhydroxyalkanoates.” *Journal of Applied Microbiology* 102 (6): 1437–49. doi:10.1111/j.1365-2672.2007.03335.x.

Villano, Marianna, Mario Beccari, Davide Dionisi, Silvia Lampis, Alfredo Miccheli, Giovanni Vallini, and Mauro Majone. 2010. “Effect of pH on the Production of Bacterial Polyhydroxyalkanoates by Mixed Cultures Enriched under Periodic Feeding.” *Process Biochemistry* 45 (5). Elsevier Ltd: 714–23. doi:10.1016/j.procbio.2010.01.008.

Vjayan, T, and V M Vadivelu. 2017. “Effect of Famine-Phase Reduced Aeration on Polyhydroxyalkanoate Accumulation in Aerobic Granules.” *Bioresource Technology*. doi:10.1016/j.biortech.2017.09.038.

3

THE LINK OF FEAST-PHASE DISSOLVED OXYGEN (DO) WITH SUBSTRATE COMPETITION AND MICROBIAL SELECTION IN PHA PRODUCTION

SUMMARY: Aeration requires large amounts of energy in PHA production by mixed microbial cultures (MMCs), particularly during the feast phase due to substrate uptake. The objective of this study was to investigate the impact of DO concentrations on microbial selection, substrate competition and PHA production performance by MMCs. This represents the first study investigating DO impact on PHA production while feeding the multiple volatile fatty acids (VFAs) typically encountered in real fermented feedstocks, as well as the substrate preferences at different DO levels. Efficient microbial cultures were enriched under both high (3.47±1.12 mg/L) and low (0.86±0.50 mg/L) DO conditions in the feast phase containing mostly the same populations but different relative abundance. The most abundant microorganisms in the two MMCs were *Plasticicumulans*, *Zoogloea*, *Paracoccus*, and *Flavobacterium*. Butyrate and valerate were found to be the preferred substrates as compared to acetate and propionate regardless of DO concentrations. In the accumulation step, the PHA production efficiency and uptake rates of butyrate and valerate were less impacted by DO change when using the culture selected under low DO in the feast phase. A high DO level leads to maximal PHA accumulation rates with the four VFAs (acetate, propionate, butyrate and valerate) present, however, if butyrate and valerate were the dominant VFAs in fermented wastewater, a low DO level would be sufficient for high PHA accumulation rates.

Key words: Polyhydroxyalkanoates (PHAs); substrate competition; DO impact; mixed microbial cultures (MMCs); microbial community analysis

Published as:
Wang Xiaofei, Oehmen Adrian, Freitas Elisabete B, Carvalho Gilda, Reis Maria A M. 2017. "The Link of Feast-Phase Dissolved Oxygen (DO) with Substrate Competition and Microbial Selection in PHA Production." Water Research 112. Elsevier Ltd: 269–78. doi:http://dx.doi.org/10.1016/j.watres.2017.01.064.

3.1 Introduction

In order to reduce the costs of PHA production by MMCs, industrial wastes/by-products were used as feedstocks, such as olive oil mill effluent (D. Dionisi et al. 2005), sugar cane molasses (Albuquerque et al. 2007) and paper mill wastewater (Bengtsson et al. 2008), through a 3 stage PHA production process as stated in section 2.4. The enrichment of PHA accumulators depends on the operational conditions applied to the selection reactor. Many studies were conducted in recent years to investigate the impact of operational conditions on culture selection and PHA accumulation, such as pH (Villano et al. 2010), organic loading rates (OLRs) (Dionisi et al. 2006), and feeding frequency (Valentino et al. 2014; Jiang et al. 2011). Compared to these operational conditions, oxygen input is more directly related to process cost, since a large amount of energy is required through the aerobic process of PHA production. However, only a few studies can be found linking the impact of dissolved oxygen (DO) on the PHA production process by mixed microbial cultures. Third et al. (2003) observed that in the feast phase of sequencing batch reactor (SBR) operation fed with acetate, biomass growth was enhanced by excessive aeration rate, yet the PHB conversion yield was improved at low DO level, since more ATP is required for cell growth than polymer synthesis. Moralejo-Gárate et al. (2013), using glycerol as substrate, fostered the culture with higher production of polyglucose over PHB under limited oxygen supply. Polyglucose can be produced from glycerol at a lower energy expense as compared to PHB. Both studies demonstrated that with different amounts of oxygen supply, MMCs consumed substrates through the pathways with lowest energy requirement. However, these works only focused on individual substrates. With the wide use of fermented industrial wastewater which contains multiple VFAs (acetate, propionate, butyrate and valerate), substrate composition becomes a crucial factor determining microbial communities enrichment and PHA production performance (Albuquerque et al. 2013). A recent study of Coats et al. (2016) investigated the impact of aeration on PHA accumulating culture selection by applying fermented dairy manure as carbon sources. They found that different aeration strategies (K_La 4, 8, 12 and 20 hr^{-1}) had no statistically significant impact on PHA synthesis rates. Thus far, no studies have investigated the link between the DO level supplied and the substrate uptake preferences by MMCs.

The amount of energy (ATP) for the conversion of each VFA into PHA is different. The metabolism of PHA production is dependent on the substrate composition, as pointed out in Table 3-1. Both acetate and butyrate are converted to acetyl-CoA, then to PHB. Propionate is converted to propionyl-CoA, then to PHV by binding with acetyl-CoA produced either from other VFAs or through decarboxylation from propionyl-CoA, or to PH2MV by coupling 2 propionyl-CoA. Valerate is converted to both acetyl-CoA and propionyl-CoA, then to PHV (Dias et al. 2008; Pardelha et al. 2012). Each mole of VFAs needs 2 moles ATP for PHA synthesis: 1 mole ATP for active transport, and another mole of ATP for intracellular conversion (Dias et al. 2008). Therefore, VFAs with longer carbon chains, like butyrate and valerate, need less ATP to produce one C-mol of PHAs as compared to acetate and propionate.

Reaction	Stoichiometry
Acetate → Acetyl-CoA	$CH_2O + ATP \rightarrow CHO_{0.5} + 0.5 \cdot H_2O$
Propionate → Propionyl-CoA	$CH_2O_{2/3} + 0.67 \cdot ATP \rightarrow CH_{4/3}O_{1/3} + 0.33 \cdot H_2O$
Butyrate → Acetyl-CoA	$CH_2O_{0.5} + 0.5ATP \rightarrow CHO_{0.5} + 0.5 \cdot NADH_2$
Valerate → Acetyl-CoA + Propionyl-CoA	$CH_2O_{2/5} + 0.4 \cdot ATP \rightarrow 0.4CHO_{0.5} + 0.6CH_{4/3}O_{1/3} + 0.4 \cdot NADH_2$

Table 3-1 VFAs uptake stoichiometry for the production of PHA precursors, on a C-mol basis

In this work the impact of DO level on MMC selection, and on substrate preference was studied. The rationale for the latter was based on the hypothesis that with less oxygen supplied (less ATP generation), valerate and butyrate will be preferred as substrates than acetate and propionate due to a lower ATP requirement. Thus, the selection reactor was operated at different DO levels during the feast phase, when substrate uptake competition takes place. PHA production kinetics in the PHA accumulation stage was determined for the different selected cultures to assess substrate competition at different DO levels.

3.2 Materials and methods

3.2.1 Experimental setup

The experimental setup consisted of two bench-scale reactor systems. The selection of PHA-accumulating cultures was carried out in two sequencing batch reactors (SBRs), each with 2L working volume, subjected to the feast and famine regime. SBR1 was operated under higher DO conditions in the feast phase (3.47±1.12 mg/L) while SBR2 was operated at a lower DO level in the feast phase (0.86±0.50 mg/L). The cultures selected were then inoculated to batch reactors for PHA accumulation testing the impacts of different DO concentrations on substrate competition and process performance of each selected culture.

Culture selection

The two SBRs were inoculated with activated sludge from the Chelas WWTP in Portugal. The 12h working cycle of both SBRs consisted of four periods, feeding (10min), aeration (11h), settling (40min) and withdrawal (10min). In the feeding periods, 50ml of mixed VFAs (25% C-mol based acetic acid, propionic acid, butyric acid and valeric acid) were fed as carbon sources, together with 0.9 L mineral nutrients medium composed of (per liter of deionised water): $MgSO_4.7H_2O$ (670mg), EDTA (110mg), $CaCl_2.2H_2O$ (80mg), K_2HPO_4 (48mg), KH_2PO_4 (38mg) and 1ml of trace elements solution ($FeCl_3.6H_2O$: 1.5 g/L, H_3BO_3: 0.15 g/L, $CuSO_4.5H_2O$: 0.03 g/L, KI: 0.03 g/L, $MnCl_2.4H_2O$: 0.12 g/L, $Ma_2MoO.2H_2O$: 0.06 g/L, $ZnSO_4.7H_2O$: 0.12 g/L and $CoCl_2.6H_2O$: 0.15 g/L) (Serafim et al. 2008). Thiourea (10 mg/L) was also added in the medium to inhibit nitrification. 50 mL of an NH_4Cl solution (4.28g/L) was added 2 hours after the start of the cycle to increase the selective pressure for PHA accumulators (Oliveira et al. 2016). The C/N/P ratio was kept at 100:8:1. The pH of each feed was adjusted to 6.5 before being added to the SBRs. The hydraulic retention time (HRT) of each SBR was kept at one day.

Air was supplied to both SBRs by air pumps through ceramic diffusers. Stirring was kept at 200 rpm by overhead stirrers (VELP-BS). pH was controlled <8 by dosing HCl (0.5M), where the actual pH varied from 7-8. Both the reactors were kept at 22 ºC by a water bath. The cycle operations included feeding and withdrawal by peristaltic pumps, aeration and stirring, which were automatically controlled by a software developed in our research group that was also used in other studies (Carvalheira et al. 2014). The software acquired pH and DO data at the same time in order to manipulate the acid dosing pump and air input valves. On/off valves were connected between air pumps and reactors, which were controlled by the software to keep the DO in the SBRs at the designated values according to the DO data acquired. The average DO level in SBR1 was 3.47±1.12 mg/L in the feast phase and 3.78±1.00 mg/L in the famine phase; while in SBR2, it was 0.86±0.50 mg/L in the feast phase and 2.42±1.26 mg/L in the famine phase. The end of the feast phase of each cycle in both SBRs was indicated by the change in DO profile due to the decreased oxygen consumption rate during the famine phase.

Different start-up strategies were applied to the two SBRs. The organic loading rate (OLR) of SBR1 was set at 50 Cmmol/L/d from the beginning of the study, which is in the range of the highest PHA-storing capacity by MMCs (Albuquerque et al. 2010). Meanwhile, under lower DO conditions, the initial OLR of SBR2 was set at 12.5 Cmmol/L/d, and then increased to 50 Cmmol/L/d gradually over 22 days. When the OLR was lower in SBR2 during the startup phase, the phosphate and ammonia concentrations in the feed were also reduced in order to keep the same C/N/P ratio. In SBR1, the sludge retention time (SRT) was kept at 4 days by purging the biomass at the end of each cycle. The SRT of SBR2 was set at 8 days initially. After the OLR of SBR2 reached the same level as SBR1, the SRT of SBR2 was decreased to 4 days gradually. After 64 days, both SBRs were operated under the same conditions. The different start-up strategies in each SBR were designed based on a previous unsuccessful attempt to enrich PHA accumulating organisms under low DO levels and identical SRT and OLR as that applied to the high DO reactor (data not shown).

PHA accumulation

After the cultures of PHA accumulating microorganisms were selected in both SBRs, four batch tests were carried out in order to study the impact of DO on the PHA accumulation process using the well selected cultures in both SBRs on day 86-89. Two DO levels were set in the accumulation tests: a high DO level: 3.47±1.12 mg/L; and a low DO level: 0.86±0.50 mg/L. Two batch reactors (500 mL) inoculated with the culture enriched in SBR1 were operated at both high DO (B1) and low DO levels (B2); while another two batch reactors (500 mL) (B3 and B4) were operated under the same high and low DO conditions inoculated with the culture enriched in SBR2. Mixed VFAs (the same composition as used for culture selection) were fed to each batch reactor using the same F/M ratio applied in the culture selection SBRs. A pulse-wise feeding strategy with 6 pulses was applied to each batch test. Ammonia was limited (no ammonia input, almost no residual ammonia) in all batches to maximize PHA production. Air was supplied by air pumps through a tubular diffuser; the DO concentrations were detected by DO sen-

sors and controlled by on/off valves through the same software used for culture selection. Agitation was supplied by magnetic stirring. pH was recorded and controlled under 8 by dosing HCl (0.5M).

3.2.2 Analytical procedures

Cell dry weight was determined by volatile suspended solids (VSS) measurements described in standard methods (APHA 1999). Ammonia and phosphate concentrations were measured by a segmented flow analyser through the Skalar San++ system.

The contents of four volatile fatty acids (VFAs): acetate, propionate, butyrate and valerate were quantified by high performance liquid chromatography (HPLC) using a Chromaster VWR Hitachi equipped with both RI and UV (wavelength 210nm) detectors and an Aminex HPX-87H (Biorad) column. 0.01N H_2SO_4 was used as the eluent at a flow rate of 0.5 mL/min with an operational temperature of 30 ºC. Samples were filtered through 0.2 µm membranes before injection. The concentrations of VFAs were calibrated through a standard curve with 15-1000 mg/L organic acids. Polyhydroxyalkanoates (PHAs) were determined by gas chromatography with flame ionization detector (GC-FID) according to the method described by Lanham et al. (2013). The concentrations of HB and HV were calibrated through standard curves with a solution made of a commercial co-polymer of PHB-PHV (88:12 molar) (Sigma) and heptadecane as internal standard.

Biomass samples were collected in both SBRs for culture selection at the startup phase (day 4), in the middle of selection (day 32) and at the steady state (day 78), respectively, to quantify the abundance of the bacterial species at different time of culture selection by high throughput DNA sequencing (done by the company DNASense ApS). Briefly, genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories) according to manufacturer's instructions. After quantification in a NanoDrop Spectrophotometer (Thermo Scientific), DNA samples were prepared using barcoded library adaptors (400nM) containing the bacterial primers targeting the V1-3 16S rRNA region (27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG) (Appendix A). PCR reactions run in duplicates, were pooled, and the amplicon libraries were purified using Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA). The concentration was measured by Quant-iTTM HS DNA Assay (Thermo Fisher Scientific, USA) and quality was evaluated with a Tapestation 2200, using D1K ScreenTapes (Agilent, USA). Afterwards, sequencing libraries were pooled in equimolar concentrations and sequenced on a MiSeq Illumina protocol of 600 cycles. The samples were paired end sequenced (2x301base pairs) and yielded between 4515 and 37063 reads after QC and bioinformatic processing. The raw sequencing data were processed using the research standard UPARSE workflow and data were analyzed through Rstudio using the ampvis package developed by the company DNASense ApS at Aalborg University, where next generation sequencing was performed. The resulting operational taxonomic units (OTUs) were classified following the MiDAS database. (McIlroy et al. 2015).

Principal component analysis (PCA) was done using the vegan package of the r-project (https://www.r-project.org/) with square root transformed in OTU counts, in order to visually detect the evolution of the microbial communities throughout the study and compare similarities. Each point represents the microbial community, grey dots indicate the OTUs and their distance represents the similarity between the samples. Fluorescence *in situ* hybridization (FISH) was performed on 4% paraformaldehyde-fixed biomass samples from both SBRs to validate the DNA sequencing results according to the method stated in Amann (1995). The oligonucleotide probes used are available in the database probeBase 2016 (Greuter et al. 2016), unless specified: EUBMIX (EUB338, EUB338II, EUB338III) for all bacteria, UCB823 for *Plasticicumulans*, ZRA23a for *Zoogloea*, PAR651 for *Paracoccus*, AMAR839 for *Amaricoccus*, AZO644 for *Azoarcus*, MEG983; CF319a for *Flavobacterium* and MEG1028 and MEG983 for *Meganema* (Thomsen et al. 2006). Additionally, Nile Blue staining was applied to confirm the presence and morphology of microorganisms containing PHA granules. An Olympus BX-51 epifluorescence microscope was used to observe both FISH and Nile Blue stained samples.

3.2.3 Kinetic and stoichiometric parameters

PHA content was determined by: %PHA= PHA/VSS*100 (g PHA/g VSS), where VSS includes active biomass (X) and PHA. Active biomass content was calculated by: X=VSS-PHA (g). The chemical formula of standard biomass ($CH_{1.8}O_{0.5}N_{0.2}$) was applied to calculate the biomass concentration on a Cmol basis, with a molecular weight of 24.6 g/Cmol.

VFA concentration is the sum of the four organic acids (acetate, propionate, butyrate and valerate) in Cmol/L; and PHA concentration corresponds to the sum of HB and HV in Cmol/L. The initial and maximum specific substrate uptake rates (q_{s_ini} and q_{s_max} in Cmol VFA/Cmol X/h) were calculated by the slope of VFA degradation as a function of the corresponding time period, divided by the active biomass concentration. PHA accumulation rates (q_{PHA} in Cmol PHA/Cmol X/h) were calculated by dividing the slope of PHA accumulation in the feast period per time by the active biomass concentration. Since there was no ammonia present in the feast period of culture selection and PHA accumulation, the active biomass concentration was constant, with no cell growth. The yields of PHA production per substrate consumption ($Y_{PHA/S}$) were determined by the ratio of the maximum PHA production rates per maximum substrate uptake rates.

3.3 Results and Discussion

3.3.1 Performance in the culture selection stage under different DO conditions

The impact of DO concentration on the selection of PHA-accumulating cultures was assessed in two SBRs operated at different DO levels (SBR1 with high DO of 3.47±1.12 mg/L and SBR2 with low DO of 0.86±0.50 mg/L) in the feast phase where substrate competition occurred with mixed VFAs (acetate, propionate, butyrate and valerate) as carbon sources. Compared to the feast phase, the selection pressure of DO in the famine phase of the two SBRs were less variant:

SBR1 with 3.78±1.00 mg DO/L, and SBR2 with 2.42±1.26 mg DO/L, both adequate for biomass synthesis and maintenance. Each SBR was operated over 120 days. Figure 3-1 shows the performance of the two reactors over the operational time, illustrating the variations in Feast to Famine (F/F) ratio and VSS concentration.

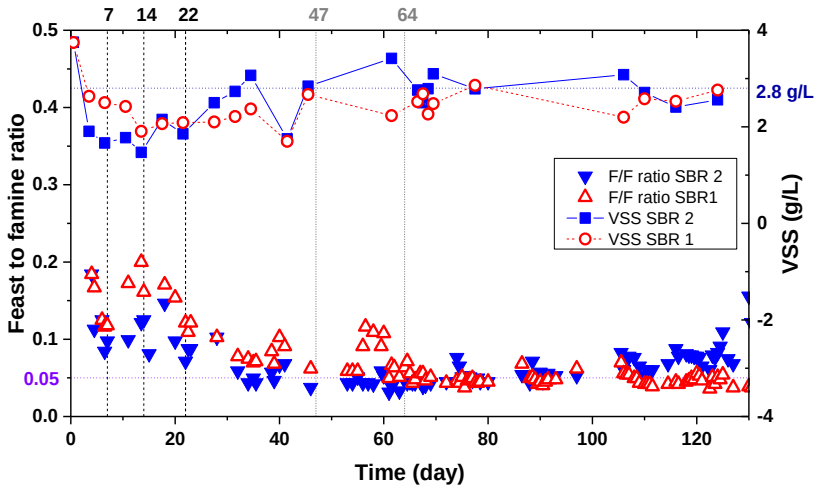

Figure 3-1 Feast to famine ratio and VSS change over time in SBR1 and SBR2 for culture selection

Under high DO conditions in the feast with a fixed OLR (50 Cmol/L/d) and SRT (4d), the biomass content in SBR1 stabilized at around 2.8 g VSS/L with a low F/F ratio at 0.050±0.008. To ensure a successful startup of SBR2, OLR was increased step-wisely with longer SRT at 8 days: 12.5 Cmol/L/d from day 1 to day 6; 25 Cmol/L/d from day 7 to day 13; 37.5 Cmol/L/d from day 14 to day 21; and 50 Cmol/L/d afterwards. After the operation was stable with the OLR of 50 Cmol/L/d, the SRT of SBR2 was decreased from 8 days to 6 days on day 47 and further reduced to 4 days on day 64. Afterwards, both reactors were operated at the same conditions, except for DO concentration. The VSS concentration stabilized at 2.8 g VSS/L with an F/F ratio of 0.048±0.009, similar to SBR1 with the same OLR and SRT at the higher DO level in the feast phase. It is suggested by Dionisi et al. (2007) that in order to select microorganisms with good capacity of storing PHAs, the feast phase should be no longer than 20% of the length of the whole cycle. In this study, both cultures under different DO conditions reached F/F ratios as low as 5%, indicating high selective pressure for PHA-storing organisms.

Figure 3-2 shows one cycle (12h) from SBR1 and SBR2 on day 67, when both SBRs were consistent in terms of operational conditions with efficient selection. All VFAs were fully exhausted and the PHA content accumulated to the maximum in the feast phase (less than an hour in both SBRs). After the addition of ammonia, PHA degradation was observed for cell growth. The specific biomass growth rate in SBR1 was 0.10±0.01 h^{-1} and in SBR2 was 0.08±0.03 h^{-1}. However, PHA was not fully consumed at the end of the cycle due to the fast depletion of ammonia in the famine phase.

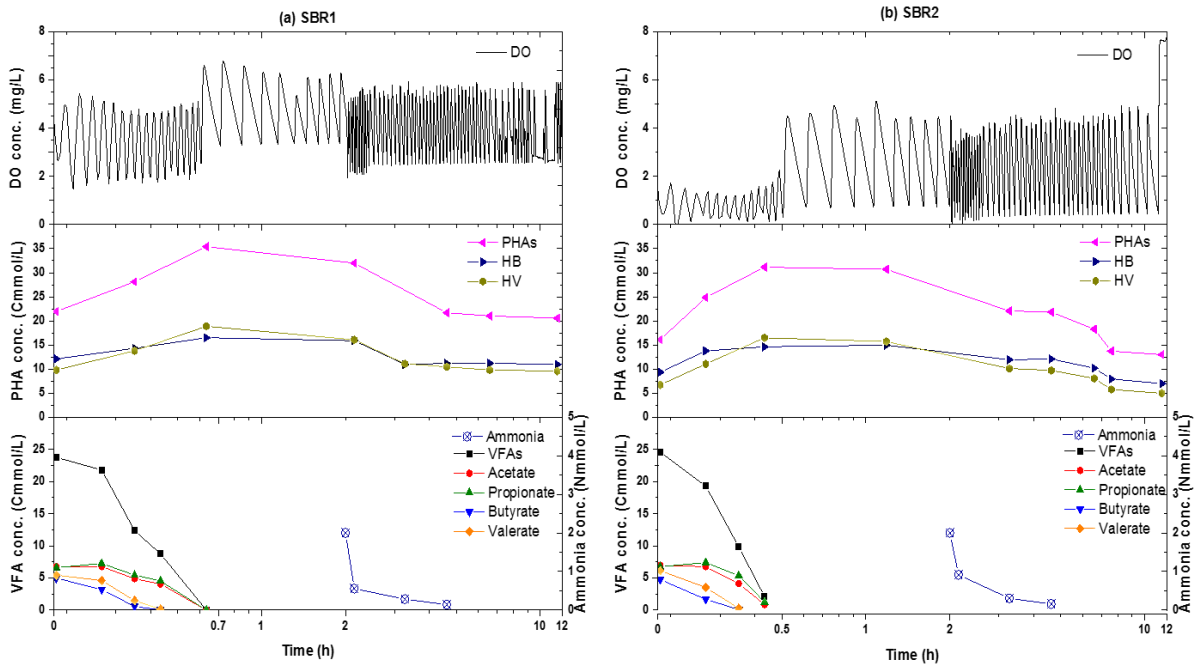

Figure 3-2 Operational cycle of culture selection in SBR1 (a) and SBR2 (b) (Time- log scale)

Table 3-2 Average performance of culture selection under different DO conditions: initial specific substrate degradation rate (qs), specific PHA accumulation rate (qPHA), PHA conversion yield (YPHA/s), and PHA composition

culture selection SBRs:			**SBR1**	**SBR2**
Rates	**Substrate consumption rate:** q_s (Cmol VFA/Cmol X/h)	$Acet_{\_ini}$	≈ 0	0.02±0.00
		$Prop_{\_ini}$	≈ 0	0.01±0.01
		$But_{\_ini}$	0.24±0.02	0.31±0.08
		$Val_{\_ini}$	0.18±0.06	0.25±0.08
		$VFAs_{\_max}$	0.87±0.09	0.92±0.02
	Polymer production rate: q_{PHA} (Cmol PHA/Cmol X/h)	$PHB_{\_max}$	0.24±0.08	0.28±0.08
		$PHV_{\_max}$	0.31±0.02	0.37±0.03
		$PHAs_{\_max}$	0.54±0.10	0.65±0.05
PHA yield and composition	**PHA production yield:** $Y_{PHA/s}$ (Cmol/Cmol)		0.62±0.05	0.71±0.06
	PHA composition: PHB:PHV (end of feast) (mol based)		47:53 (±1)	49:51 (±0.5)

Table 3-2 depicts the average performance over 3 non-consecutive days (day 67, 78 and 88) of analysis under identical and stable operational conditions of the cultures selected in each SBR. Though all four VFAs were depleted during the feast phase, butyrate and valerate were consumed faster than acetate and propionate at both feast-phase DO levels. At the beginning of the feast phase, the initial specific uptake rates of acetate and propionate were negligible in both SBRs, which indicated that the microorganisms selected preferred to uptake butyrate and valerate first, then acetate and propionate. Butyrate was also reported as preferred substrate for PHA production compared to acetate by Marang et al. (2013). It was found that when MMCs were fed by a mixture of butyrate and acetate, only after the depletion of butyrate, acetate started to be consumed.

The substrate consumption rates and polymer production rates of the selected MMCs are higher than those reported in previous studies applying fermented feedstocks (Albuquerque et al, 2010; Bengtsson et al. 2008). Probably, studies applying fermented feedstocks, although enriched, had a higher fraction of non-PHA storing population that grew at expenses of non-fermented carbon sources, resulting in lower specific rates. The PHA conversion yield of 0.6 - 0.7 Cmol PHA/Cmol VFA (Table 3-2) are in the range of values reported in previous studies of PHA production fed by synthetic (acetate) (Johnson et al. 2009; Serafim et al. 2004), or fermented feedstocks (Bengtsson et al. 2008; Coats, Watson, and Brinkman 2016). Similar HB:HV ratios were achieved in both reactors since the same substrate composition was applied, which has been shown to be the main factor influencing PHA composition (Pardelha et al. 2014). In the study of Coats et al. (2016), a similar HB: HV ratio was observed with different KLa applied in the enrichment reactors. They also pointed out that the reduced aeration rate had limited impact on both VFA consumption and PHA production. Even though different KLa were tested in their study, the average DO in the feast phase was at the same level in each reactor, which could be the main reason for the similarity of performance in their reactors. Generally, better performance was observed under low DO conditions (SBR2) in this study, in terms of slightly higher substrate consumption rates, PHA production rates, and PHA conversion yields (Table 3-2). Given that in the feast phase cell growth was precluded by the absence of ammonia, all the consumed carbon was directed to cell maintenance and PHA synthesis. At the low DO level, the microbial communities that required less ATP and carbon for maintenance might be selected, with more carbon substrate being converted to PHAs. In the famine phase, during the period of ammonia presence, PHAs were consumed for both cell growth and maintenance. The same amount of ammonia was depleted for cell growth in both reactors, since the DO levels in SBR2 during the famine phase was not likely to limit the growth of microorganisms, thus the same amount of C-mol of biomass was produced in both SBRs. Nevertheless, the PHA consumption rate in the famine phase of SBR1 was statistically slightly higher than that of SBR2, which suggested that the culture selected in SBR2 required less ATP and carbon for cell maintenance. Previous studies have also shown that lower energy requirements for cell maintenance purposes (or lower cell decay rates) are associated with a lower abundance or energetic efficiency of elec-

tron acceptor (e.g. oxygen or nitrate), which has been incorporated into mathematical models (Hauduc et al. 2013). This effect is in agreement with the results found in the present study, and can likely be explained by the stringent response, whereby the energy requirement of cells varies to conditions with different abundance of electron acceptors, like oxygen. (van Loosdrecht & Henze 1999).

3.3.2 Microbial community characterization under different DO conditions

The microorganisms selected in SBRs under different DO concentrations were characterized by high throughput amplicon sequencing of the 16S rRNA gene. At the startup phase, the populations of each SBR were characteristic of non-selected activated sludge (shown in Appendix A-1). High similarity of the microbial communities in both SBRs on day 4 is shown by the principle component analysis (PCA) in Figure 3-3.

The mid-point of culture selection (day 32) revealed greater differences in the microbial communities (Figure 3-3): in SBR1, the most abundant populations were *Paracoccus* (14.2%), unidentified *Proteobacteria* (o__DB1−14_OTU_14) (11.7%) and *Plasticicumulans (10.6%);* while SBR2 was dominated by totally different organisms: *Amaricoccus* (26.8%), *Azoarcus* (12.3%) and a different OTU of unidentified *Proteobacteria* (o__DB1−14_OTU_17) (8.7%) (The most abundant bacteria in both SBRs on day 32 are shown in Appendix A-2). Besides the different DO concentrations, the two SBRs on day 32 were operated under different SRTs: 4d for SBR1 and 8d for SBR2. As discussed in other studies, SRT is a strong selective pressure for PHA accumulating microorganisms. Lemos et al. (2008) reported that *Amaricoccus* and *Azoarcus* were favored at long SRT (10d), and that when shortening the SRT to 1d, *Amaricoccus* was almost completely washed out. However, *Plasticicumulans* were commonly found as the dominant bacteria in reactors operated at short SRTs (Johnson et al. 2009). The difference in populations on day 32 in this study was likely due to the different SRTs in the two SBRs.

Indeed, many of the most abundant OTUs were common in both SBRs and differed only in their relative abundance, although some of them were undetected or detected only in small numbers in the inoculum (results not shown). PCA also showed high similarity of the cultures selected on day 78 (Figure 3-3). In SBR2, *Amaricoccus* and *Azoarcus* that used to be present on day 32 were removed by the decrease of SRT from 8d to 4d. In the meanwhile, *Plasticicumulans* took over as the most abundant community. Compared to SRT, DO had less impact on microbial selection, but still influenced the relative abundance of the common populations, showing that the competition for oxygen between the selected groups of organisms in the two cultures occurred. On day 78, the bacteria selected with higher abundance (each with >10% of total read count) in SBR1 were *Plasticicumulans, Zoogloea and Paracoccus,* while in SBR2 were *Plasticicumulans, Zoogloea* and *Flavobacterium* (Table 3-3). At this stage, almost 70% of the total amplicon count under both DO conditions corresponded to microorganisms that have been reported as PHA storers. Positive signals of PHA granules were observed from Nile Blue staining in the majority of the bacteria selected.

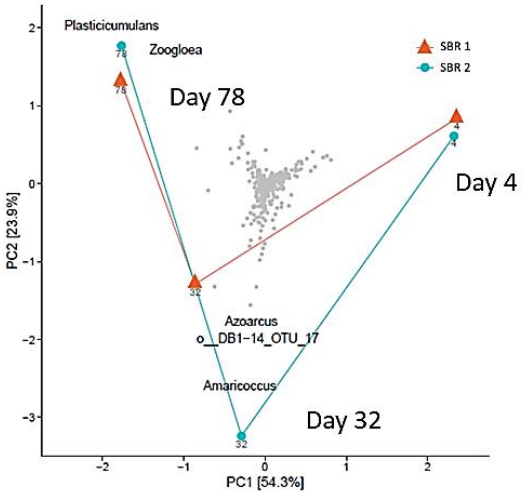

Figure 3-3 PCA results of microbial communities selected in SBR1 and SBR2

Plasticicumulans comprised 27.6% of total bacteria in SBR1 and 50.6% in SBR2 (confirmed by FISH in Figure 3-4 (a) and (b)). This organism has been reported as the dominant population in acetate fed cultures, which could adapt very well to other VFAs (Johnson et al. 2009; Marang et al. 2013). *Zoogloea,* the second most abundant bacteria in both SBRs, are known as PHB producers at various temperature ranges (Jiang et al. 2011). *Paracoccus,* has also been described as a PHA-storer, with a strong preference for taking up propionate, butyrate and valerate (Albuquerque et al. 2013). This organism was enriched with higher abundance in SBR1. *Meganema* was also an important organism in this study, particularly in SBR1 (6.7%). It was previously described as a PHA accumulator (Kragelund et al. 2005; Coats, Watson, and Brinkman 2016) and identified in a mixed microbial community for PHA production (Dionisi et al. 2005). This study showed strong evidence that this organism is enriched in a PHA selection MMC reactor with high capacity of PHA storage (see FISH (c) and Nile Blue (d) pictures in Figure 3-4). Even though *Meganema* was often reported as responsible for bulking in industrial wastewater treatment plants (Levantesi et al. 2004), no settling problems occurred in this study. *Flavobacterium* were commonly found in the activated sludge from WWTPs, as well as the main microorganisms existent in the cultures selected for PHA production (Dionisi et al. 2005). However, the capacity of PHA storage of a *Flavobacterium* was only reported once by Tezuka (1969); recent studies showed the presence of this genus in the enriched MMCs for PHA production but the function was rarely discussed. *Amaricoccus*, *Azoarcus* and *Brevundimonas*, which were reported with the capacity of PHA storage in various studies, were identified with lower proportions in both reactors (Lemos et al. 2008; Silva et al. 2007).

Table 3-3 The 15 most abundant bacteria identified by 16S rRNA gene amplicon sequencing of the cultures enriched in the two SBRs on day 78 of culture selection.

	SBR1			SBR2		
	PHYLUM	GENUS	%	PHYLUM	GENUS	%
1	Proteobacteria	***Plasticicumulans***	27.6	Proteobacteria	***Plasticicumulans***	50.6

2	Proteobacteria	***Zoogloea***	19.7	Proteobacteria	***Zoogloea***	13.5
3	Proteobacteria	***Paracoccus***	13.0	Bacteroidetes	*Flavobacterium*	12.0
4	Proteobacteria	***Meganema***	6.7	Proteobacteria	*Hyphononadaceae**	5.5
5	Proteobacteria	*Alphaproteobacteria****	4.8	Proteobacteria	***Paracoccus***	2.5
6	Firmicutes	*Lactobacillus*	4.3	Proteobacteria	***Azoarcus***	0.8
7	Proteobacteria	*Aminobacter*	3.3	Proteobacteria	*Aminobacter*	0.7
8	Bacteroidetes	*Flavobacterium*	2.4	Proteobacteria	***Brevundimonas***	0.7
9	Proteobacteria	***Amaricoccus***	2.0	Proteobacteria	*Alphaproteobacteria****	0.5
10	Proteobacteria	*Hyphononadaceae**	1.2	Proteobacteria	*Aquimonas*	0.4
11	Proteobacteria	*Aquimonas*	0.9	Proteobacteria	***Meganema***	0.3
12	Proteobacteria	***Brevundimonas***	0.3	Proteobacteria	***Amaricoccus***	0.2
13	Proteobacteria	*Dokdonella*	0.3	Firmicutes	*Lactobacillus*	0.1
14	Proteobacteria	***Azoarcus***	0.1	Proteobacteria	*Dokdonella*	0.1
15	Proteobacteria	*Rhizobiales***	0.1	Bacteroidetes	*Cytophagaceae**	0.1
	Total Bacteria reported as PHA storers (in **Bold**)		69.4	Total Bacteria reported as PHA storers (in **Bold**)		68.6

**Identified only to the family level; **Identified only to the order level; ***Identified only to the class level*

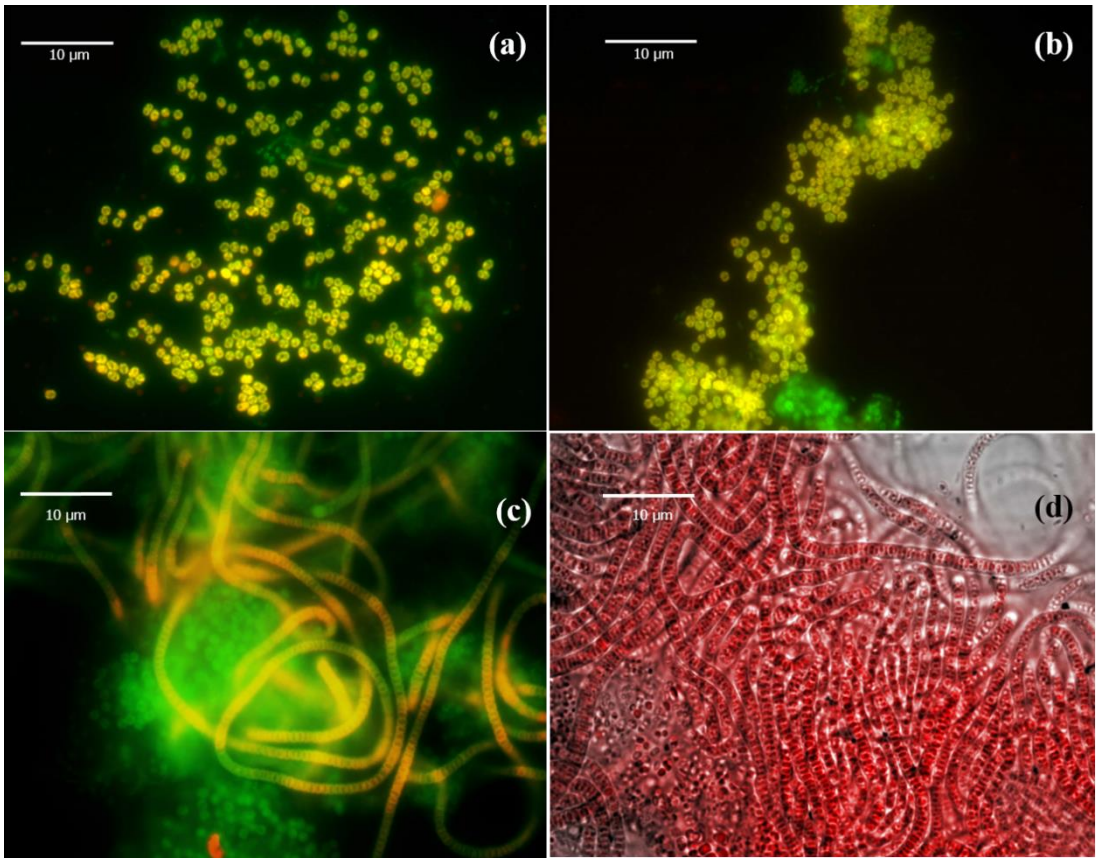

Figure 3-4 *Plasticicumulans* in SBR1 (a) and SBR2 (b) detected by FISH: UCB823-targeted bacteria (in yellow) are *Plasticicumulans*; all other bacteria are in green; *Meganema* in SBR1 (c) by FISH: MEG983-targeted bacteria (in yellow) are *Meganema*; all other bacteria are in green; and *Meganema* in SBR1 (d) by Nile Blue staining: PHA granules are in red.

As the differences and similarities associated with the community structure of MMCs were identified in both SBRs, it indicated that at each DO level tested, efficient PHA accumulating communities were selected with different startup strategies, but with similar PHA productivity and process performance.

3.3.3 PHA accumulation performance at different DO levels

Even though low DO conditions in the feast phase proved to have no significant effects on the selection of PHA accumulators in MMCs, the impact of DO on the PHA accumulation stage still required further study. Four batch tests (B1, B2, B3 and B4) were designed to study the DO impact on PHA accumulation performance using the cultures selected in the two SBRs. The average kinetics and yields related to PHA production of the first three pulses in each batch test are shown in Table 3-4. The HB: HV ratio was calculated by the average of the net polymer production of the first 3 pulses, while maximum PHA storage was determined at the end of the 6^{th} pulse.

Table 3-4 Average performance of the first three pulses in each batch test using the two cultures selected under high and DO conditions

	Batch tests	**B1**	**B2**	**B3**	**B4**
	DO Condition	**high DO**	**low DO**	**high DO**	**low DO**
	Culture source	**SBR1 (High DO in feast)**		**SBR2 (Low DO in feast)**	
Kinetics	**q_{s_max} (Cmol VFA /C-mol X/h)**	1.19±0.02	0.45±0.18	1.24±0.17	0.66±0.03
	q_{PHA} (Cmol PHA/C-mol X/h)	1.20±0.15	0.33±0.14	1.18±0.25	0.66±0.07
PHA yield and composition	**$Y_{PHA/s}$ (Cmol PHA/Cmol VFA)**	1.00±0.11	0.71±0.03	0.94±0.08	1.00±0.04
	HB:HV (mol: mol)	41:59 (±3.9)	38:62 (±1.7)	41:59 (±0.6)	41:59 (±1.4)
	Maximum PHA storage (% w/w)	69%	49%	65%	60%

DO had a significant impact on PHA accumulation by the culture selected under high DO conditions in the feast (see B1 and B2). Substrate uptake rate, PHA accumulation rate, PHA conversion yield and PHA storage capacity were reduced with the decrease of DO concentration. Meanwhile, when using the culture selected under low DO conditions in the feast for PHA accumulation (see B3 and B4), DO had little impact on PHA yield and storage capacity, however, the substrate uptake rate and PHA accumulation rate were far lower at the low DO level (B4). Indeed, both rates were only about half of those observed under high DO concentrations. These results indicated that a high DO level was required for efficient PHA accumulation when feeding the 4 VFAs, no matter which MMC was used. Under high DO conditions, both cultures (see B1 and B3) achieved approximately equal rates, high PHA contents (between 65% to 69% w/w) and similar PHA conversion yields.

In these PHA accumulation tests, cell growth was precluded by the absence of ammonia, and all VFAs consumed were used for cell maintenance and PHA synthesis. The PHA conversion yield and PHA storage capacity of the culture selected in SBR1 and subjected to low DO conditions (B2) were much lower than the other assays. The maintenance rates were calculated by the PHA

consumption rates in the end of each batch test after the depletion of VFAs. The comparatively higher maintenance rate of B2 (0.075±0.001 mol/Cmol/h) compared to other batch tests (0.011±0.007 mol/Cmol/h in average of B1, B3 and B4) indicated that more carbon was used for cell maintenance, thus compromising the synthesis of PHA. Contrastingly, the culture selected in SBR2 showed a superior adaptability to the DO change, and the maximum PHA storage was not impacted by DO conditions (see B3 and B4). This fact showed that microbial organisms selected at low DO levels in the feast phase required less oxygen to produce the same amount of polymers since less energy is required for maintenance. The HB: HV ratio (41:59) in B1, B3 and B4 was rather close to the theoretical ratio (HB: HV=1:2) without considering decarboxylation and cell maintenance. Nevertheless, the maintenance rates in these batches were lower than the commonly used value (0.02 mol/Cmol/h) for metabolic modelling in other studies (Dias et al. 2005; Pardelha et al. 2014). Hence, high PHA conversion yields were obtained by excluding cell growth and the low energy requirement for decarboxylation and maintenance.

As shown in Figure 3-5, the specific oxygen uptake rate (SOUR) of each batch test decreased along pulses (except for B1 and B3 in the first pulse). Generally, SOURs of high DO batches (B1 and B3) were significantly higher than low DO assays (B2 and B4), which also suggested that high DO conditions were necessary to maximize the biomass activities. The lowest SOUR was observed in B2. The activity of microorganisms selected under high DO conditions in the feast might be limited by the lower air supply provided in this test.

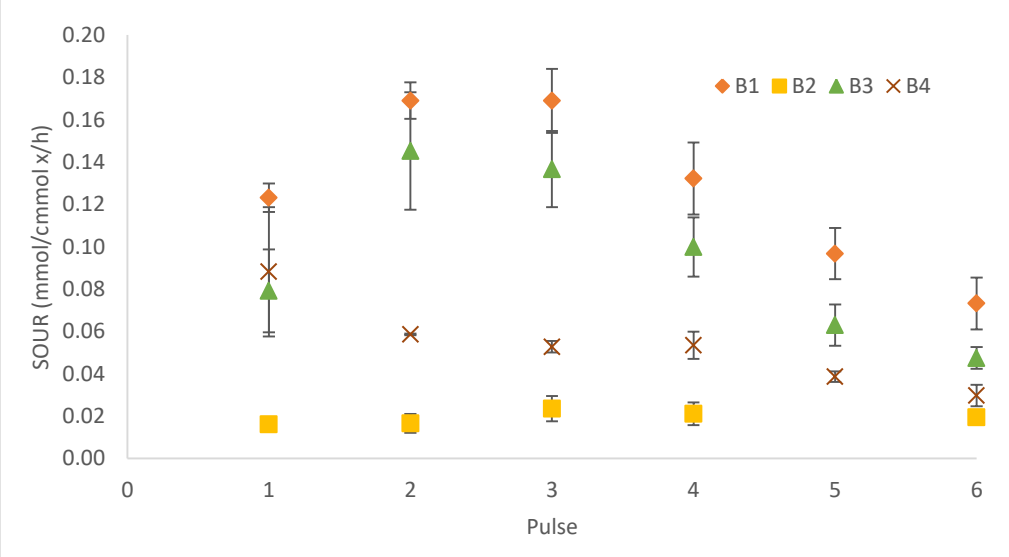

Figure 3-5 SOUR of each of the 6 pulses in the 4batch tests

The impact of DO on PHA accumulation and biomass growth was also studied by Pratt et al. (2012) when feeding a synthetic mixture of acetate and propionate in batch accumulation reactors, to the biomass selected in SBRs fed by fermented dairy waste under high DO levels. They obtained the same PHA capacity of around 35% w/w under both high (5.5 mg/L) and low DO (0.2 mg/L) conditions, and found that the PHA accumulation rate was faster under low DO at

the beginning of the experiment. Since ammonia was added in their accumulation tests, there was a competition for substrate between the PHA accumulation and cell growth processes. Under low DO conditions, growth was restricted but PHA production was not impacted, given that cell growth required more energy than PHA synthesis. However, in our study, no biomass growth took place and all substrate was channeled for PHA synthesis and cell maintenance, which led to the different results of both studies. Vargas et al. (2014) also conducted batch tests for PHA accumulation under different DO levels (0.5 mg/L, 3.0 mg/L and 5.5 mg/L), the first two DO concentrations were comparable to the low DO level (0.86±0.50 mg/L) and high DO level (3.47±1.12 mg/L) set in our experiment. Similar to our results, they observed higher PHA storage capacity under higher DO (3.0 mg/L) than lower DO (0.5 mg/L) in the accumulation stage. However, the PHA production rate and yield were not influenced by DO change in their work. Since only acetate and propionate were fed as carbon source for both culture selection and PHA accumulation in their study, the microorganisms enriched might have higher affinity to acetate and propionate regardless of DO change.

3.3.4 The impact of DO on substrate competition

The preferences for each VFA in the 4 accumulation tests were also analyzed. The average uptake of each VFA in the first 3 pulses of each batch test is shown in Figure 3-6. Butyrate and valerate started to be consumed immediately after feeding, while acetate and propionate had a lag phase, which indicated that the presence of butyrate and valerate at high concentrations might inhibit the consumption of acetate and propionate. The initial substrate uptake rates (q_{s_ini}) (Table 3-5) calculated by the average slopes of VFA consumption under both DO conditions confirmed these results. Compared to butyrate and valerate, acetate and propionate had much lower initial degradation rates in each assay, nearly zero at the beginning of each pulse. This observation seemed to indicate that regardless of DO concentration, butyrate and valerate are preferred as substrates compared to acetate and propionate for both PHA storing microorganisms' selection and PHA accumulation, likely since less ATP is required for taking up 1 Cmol of these longer-chain VFAs.

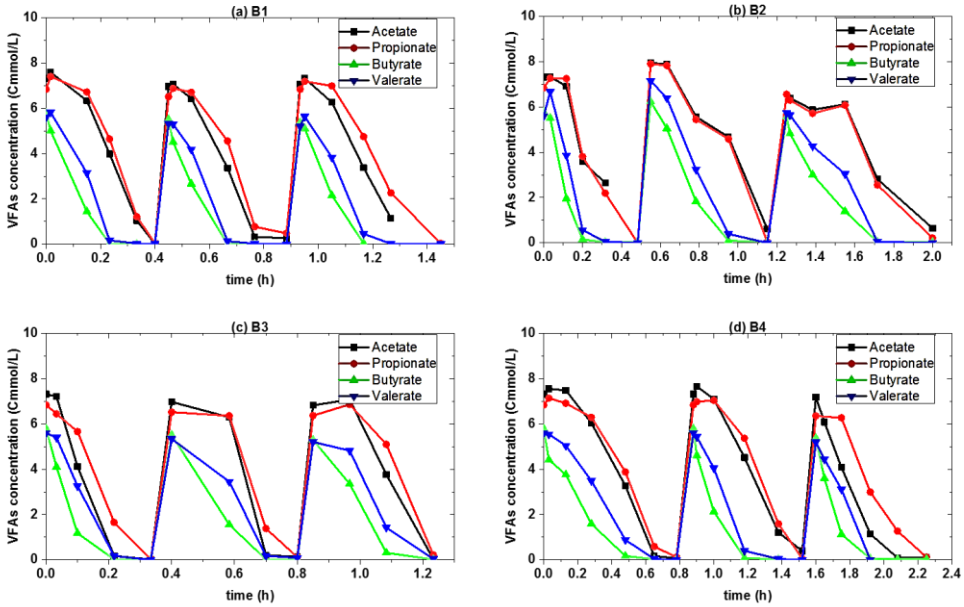

Figure 3-6 VFA consumption in the first 3 pulses of each batch test

Table 3-5 Average initial and maximum substrate uptake rates of the 4 VFAs in the first 3 pulses of each batch test

	Initial substrate consumption rate: q_{s_ini} (C-mol /C-mol X/h)			
	Acetate	Propionate	Butyrate	Valerate
B1	0.11 ±0.01	0.01 ±0.02	0.44 ±0.03	0.23 ±0.02
B2	0.03 ±0.02	0.01 ±0.00	0.20 ±0.03	0.16 ±0.05
B3	0.04 ±0.00	0.08 ±0.07	0.35 ±0.15	0.21 ±0.07
B4	0.06 ±0.01	0.01 ±0.02	0.36 ±0.15	0.18 ±0.05
	Maximum substrate uptake rates q_{s_max} (C-mol / C-mol X/h)			
	Acetate	Propionate	Butyrate	Valerate
B1	0.39 ±0.02	0.38±0.04	0.44±0.03	0.38±0.01
B2	0.13 ±0.02	0.13±0.02	0.20 ±0.03	0.16±0.06
Δ (B1, B2)	Δ Acetate 67%	Δ Propionate 66%	Δ Butyrate 54%	Δ Valerate 58%
B3	0.50 ±0.12	0.34±0.02	0.37±0.14	0.26±0.07
B4	0.23±0.05	0.23±0.01	0.36±0.11	0.21±0.05
Δ (B3, B4)	Δ Acetate 54%	Δ Propionate 33%	Δ Butyrate 3%	Δ Valerate 16%

Acetate and propionate uptake rates increased significantly after butyrate and valerate were partially consumed (Figure 3-6). The comparison of the maximum substrate rate of the individual VFAs under both DO levels further reveals the impact of DO on the overall substrate consumption kinetics in the PHA accumulation stage. The maximum substrate uptake rates (q_{s_max}) were calculated by the average maximum slopes during the first three pulses; and the difference (Δ) of maximum substrate uptake rate between the two operational DO conditions of the same selected culture was calculated by the percentage of the difference between the low and high DO batch tests (Table 3-5).

DO had a more significant impact on the maximum degradation rate of each individual VFA for the cultures selected in SBR1 vs. SBR2. Comparing B2 to B1, both using the cultures selected in SBR1, the q_{s_max} of each VFA decreased by more than 50% after reducing the DO concentration. The SBR1 culture probably had an overall low affinity for oxygen, thus oxygen content highly affected the substrate consumption rates. However, for the culture selected in SBR2, large reductions (>30%) of q_{s_max} when decreasing the DO concentration were only observed for acetate and propionate uptake. The impact of DO on the maximum butyrate and valerate consumption rates was comparatively lower. Only a 3% decrease was observed for butyrate and 16% for valerate uptake at the low DO level as compared to the high DO level. The low DO level of the feast phase in the selection reactor might have fostered a microbial community that could consume butyrate and valerate at close to their maximum uptake rates with less oxygen input. Thus, the affinity of the low DO culture for butyrate and valerate uptake at low DO levels was probably higher as compared to acetate or propionate uptake, and even more than that for the high DO culture. These facts confirmed the hypothesis that the DO could be a driving force for culture selection, leading to a higher abundance of the microorganisms that prefer to take up butyrate and valerate than acetate and propionate under low DO conditions.

The observations from the batch tests for PHA accumulation confirmed that DO concentration did not significantly impact substrate preference. Butyrate and valerate were preferred regardless of the DO conditions in both the culture selection and PHA accumulation stages. Nevertheless, the acetate and propionate uptake rates were lower at low DO, while butyrate and valerate uptake rates were not significantly affected by DO. Hence, high DO level is required for PHA accumulation with the four VFAs present. In anaerobic fermentation processes for VFAs production from wastewater, wastes or by-products, if the proportion of butyrate and valerate can be enhanced, for example, by controlling the operational conditions like pH (Hasan et al. 2015), it may be possible to apply these VFA products on both culture selection and PHA accumulation stages under low DO conditions, optimizing the process.

3.4 Conclusions

Efficient PHA accumulating cultures were selected at two DO levels in feast phase with different startup strategies. Compared to SRT, DO had a lower selective pressure for enriching the different cultures, but it had an impact on the abundance of the microbial communities enriched. PHA storers preferred to use butyrate and valerate rather than acetate and propionate regardless of the DO conditions of the reactors. The low DO level applied to the feast phase contributed to the selection of PHA accumulating cultures which performed better under low DO conditions for PHA production, without significant loss of process efficiency in terms of PHA production rate and yield. Since butyrate and valerate uptakes by the culture selected at low DO in the feast phase were less impacted by DO change, it would be feasible to accumulate PHA efficiently under low DO conditions if using fermented streams enriched with butyrate and valerate.

References

Albuquerque, M. G E, M. Eiroa, C. Torres, B. R. Nunes, and M. a M Reis. 2007. "Strategies for the Development of a Side Stream Process for Polyhydroxyalkanoate (PHA) Production from Sugar Cane Molasses." *Journal of Biotechnology* 130 (4): 411–21. doi:10.1016/j.jbiotec.2007.05.011.

Albuquerque, M G E, C A V Torres, and M A M Reis. 2010. "Polyhydroxyalkanoate (PHA) Production by a Mixed Microbial Culture Using Sugar Molasses: Effect of the Influent Substrate Concentration on Culture Selection." *Water Research* 44 (11): 3419–33. doi:http://dx.doi.org/10.1016/j.watres.2010.03.021.

Albuquerque, Maria G E, Gilda Carvalho, Caroline Kragelund, Ana F Silva, Maria T Barreto Crespo, Maria A M Reis, and Per H Nielsen. 2013. "Link between Microbial Composition and Carbon Substrate-Uptake Preferences in a PHA-Storing Community." *The ISME Journal* (2013) 7,1 (1751–7362/13).

Amann, Rudolf I. 1995. "In Situ Identification of Micro-Organisms by Whole Cell Hybridization with rRNA-Targeted Nucleic Acid Probes – Bookmetrix Analysis." In *Molecular Microbial Ecology Manual*, edited by F. Akermans, A., van Elsas, J., de Bruijn. Springer Netherlands. http://www.bookmetrix.com/detail/chapter/c08fc37d-0782-4702-a7e2-692dd429bea7#downloads.

APHA. 1999. "Standard Methods for the Examination of Water and Wastewater." *American Public Health Association*. http://www.mwa.co.th/download/file_upload/SMWW_1000-3000.pdf.

Bengtsson, Simon, Alan Werker, Magnus Christensson, and Thomas Welander. 2008. "Production of Polyhydroxyalkanoates by Activated Sludge Treating a Paper Mill Wastewater." *Bioresource Technology* 99 (3): 509–16. doi:http://dx.doi.org/10.1016/j.biortech.2007.01.020.

Carvalheira, Mónica, Adrian Oehmen, Gilda Carvalho, Mário Eusébio, and Maria a.M. Reis. 2014. "The Impact of Aeration on the Competition between Polyphosphate Accumulating Organisms and Glycogen Accumulating Organisms." *Water Research* 66: 296–307. doi:10.1016/j.watres.2014.08.033.

Coats, Erik R, Benjamin S Watson, and Cynthia K Brinkman. 2016. "Polyhydroxyalkanoate Synthesis by Mixed Microbial Consortia Cultured on Fermented Dairy Manure : Effect of Aeration on Process Rates / Yields and the Associated Microbial Ecology." *Water Research* 106. Elsevier Ltd: 26–40. doi:10.1016/j.watres.2016.09.039.

Dias, João M.L., Luísa S. Serafim, Paulo C. Lemos, Maria a.M. Reis, and Rui Oliveira. 2005. "Mathematical Modelling of a Mixed Culture Cultivation Process for the Production of Polyhydroxybutyrate." *Biotechnology and Bioengineering* 92 (2): 209–22. doi:10.1002/bit.20598.

Dias, João M L, Adrian Oehmen, Luísa S Serafim, Paulo C Lemos, Maria A M Reis, and Rui Oliveira. 2008. "Metabolic Modelling of Polyhydroxyalkanoate Copolymers Production by Mixed Microbial Cultures." *BMC Systems Biology* 2 (1): 59. doi:10.1186/1752-0509-2-59.

Dionisi, D., G. Carucci, M. Petrangeli Papini, C. Riccardi, M. Majone, and F. Carrasco. 2005. "Olive Oil Mill Effluents as a Feedstock for Production of Biodegradable Polymers." *Water Research* 39 (10): 2076–84. doi:10.1016/j.watres.2005.03.011.

Dionisi, Davide, Mario Beccari, Simona D. Gregorio, Mauro Majone, Marco Petrangeli Papini, and Giovanni Vallini. 2005. "Storage of Biodegradable Polymers by an Enriched Microbial Community in a Sequencing Batch Reactor Operated at High Organic Load Rate." *Journal of Chemical Technology and Biotechnology* 80 (December 2004): 1306–18. doi:10.1002/jctb.1331.

Dionisi, Davide, Mauro Majone, Giovanni Vallini, Simona Di Gregorio, and Mario Beccari. 2006. "Effect of the Applied Organic Load Rate on Biodegradable Polymer Production by Mixed Microbial Cultures in a Sequencing Batch Reactor." *Biotechnology and Bioengineering* 93 (1): 76–88. doi:10.1002/bit.20683.

Dionisi, Davide, Mauro Majone, Giovanni Vallini, Simona Di Gregorio, and Mario Beccari. 2007. "Effect of the Length of the Cycle on Biodegradable Polymer Production and Microbial Community Selection in a Sequencing Batch Reactor." *Biotechnology Progress* 23 (5): 1064–73. doi:10.1021/bp060370c.

Hasan, Salah Din Mahmud, Citieli Giongo, Mônica Lady Fiorese, Simone Damasceno Gomes, Tatiane Caroline Ferrari, and Tarcio Enrico Savoldi. 2015. "Volatile Fatty Acids Production from Anaerobic Treatment of Cassava Waste Water: Effect of Temperature and Alkalinity." *Environmental Technology* 36 (20): 2637–46. doi:10.1080/09593330.2015.1041426.

Hauduc, H., L. Rieger, A. Oehmen, M.C.M. van Loosdrecht, Y. Comeau, A. Héduit, P.A. Vanrolleghem, and S. Gillot. 2013. "Critical Review of Activated Sludge Modeling: State of Process Knowledge, Modeling Concepts, and Limitations." *Biotechnology and Bioengineering* 110 (1): 24–46. doi:10.1002/bit.24624.

Hong, Chen, Li HaiBo, and Xia YunFeng. 2010. "Acclimating PHA Storage Capacity of Activated Sludge with Static Magnetic Fields." *Enzyme and Microbial Technology* 46 (7). Elsevier Inc.: 594–97. doi:http://dx.doi.org/10.1016/j.enzmictec.2010.03.004.

Jiang, Yang, Leonie Marang, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2011. "Effect of Temperature and Cycle Length on Microbial Competition in PHB-Producing Sequencing Batch Reactor." *The ISME Journal* 5 (5). Nature Publishing Group: 896–907. doi:10.1038/ismej.2010.174.

Johnson, Katja, Yang Jiang, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2009. "Enrichment of a Mixed Bacterial Culture with a High Polyhydroxyalkanoate Storage Capacity." *Biomacromolecules* 10 (4). American Chemical Society: 670–76. doi:10.1021/bm8013796.

Kragelund, Caroline, Jeppe Lund Nielsen, Trine Rolighed Thomsen, and Per Halkjær Nielsen. 2005. "Ecophysiology of the Filamentous Alphaproteobacterium Meganema Perideroedes in Activated Sludge." *FEMS Microbiology Ecology* 54 (1): 111–22. doi:10.1016/j.femsec.2005.03.002.

Lanham, Ana B., Ana Rita Ricardo, Maria G E Albuquerque, Filipa Pardelha, Mónica Carvalheira, Marta Coma, Joana Fradinho, Gilda Carvalho, Adrian Oehmen, and Maria a M Reis. 2013. "Determination of the Extraction Kinetics for the Quantification of Polyhydroxyalkanoate Monomers in Mixed Microbial Systems." *Process Biochemistry* 48 (NOVEMBER 2013): 1626–34. doi:10.1016/j.procbio.2013.07.023.

Lemos, Paulo C, Caterina Levantesi, Luisa S Serafim, Simona Rossetti, Maria a M Reis, and Valter Tandoi. 2008. "Microbial Characterisation of Polyhydroxyalkanoates Storing Populations Selected under Different Operating Conditions Using a Cell-Sorting RT-PCR

Approach." *Applied Microbiology and Biotechnology* 78 (2): 351–60. doi:10.1007/s00253-007-1301-5.

Levantesi, Caterina, Claudia Beimfohr, Bert Geurkink, Simona Rossetti, Karin Thelen, Janneke Krooneman, Jiri Snaidr, Jaap van der Waarde, and Valter Tandoi. 2004. "Filamentous Alphaproteobacteria Associated with Bulking in Industrial Wastewater Treatment Plants." *Systematic and Applied Microbiology* 27 (6): 716–27. doi:Doi 10.1078/0723202042369974.

Marang, Leonie, Yang Jiang, Mark C M van Loosdrecht, and Robbert Kleerebezem. 2013. "Butyrate as Preferred Substrate for Polyhydroxybutyrate Production." *Bioresource Technology* 142 (August). Elsevier Ltd: 232–39. doi:10.1016/j.biortech.2013.05.031.

McIlroy, Simon Jon, Aaron Marc Saunders, Mads Albertsen, Marta Nierychlo, Bianca McIlroy, Aviaja Anna Hansen, Søren Michael Karst, Jeppe Lund Nielsen, and Per Halkjær Nielsen. 2015. "MiDAS: The Field Guide to the Microbes of Activated Sludge." *Database : The Journal of Biological Databases and Curation* 2015 (January): bav062. doi:10.1093/database/bav062.

Moralejo-Gárate, Helena, Robbert Kleerebezem, Anuska Mosquera-Corral, and Mark C M van Loosdrecht. 2013. "Impact of Oxygen Limitation on Glycerol-Based Biopolymer Production by Bacterial Enrichments." *Water Research* 47 (3): 1209–17. doi:http://dx.doi.org/10.1016/j.watres.2012.11.039.

Oliveira, Catarina S.S., Carlos E. Silva, Gilda Carvalho, and Maria A. Reis. 2016. "Strategies for Efficiently Selecting PHA Producing Mixed Microbial Cultures Using Complex Feedstocks: Feast and Famine Regime and Uncoupled Carbon and Nitrogen Availabilities." *New Biotechnology*. doi:10.1016/j.nbt.2016.10.008.

Pardelha, Filipa, Maria G E Albuquerque, Maria a M Reis, João M L Dias, and Rui Oliveira. 2012. "Flux Balance Analysis of Mixed Microbial Cultures: Application to the Production of Polyhydroxyalkanoates from Complex Mixtures of Volatile Fatty Acids." *Journal of Biotechnology* 162 (2–3). Elsevier B.V.: 336–45. doi:10.1016/j.jbiotec.2012.08.017.

Pardelha, Filipa, Maria G E Albuquerque, Maria a M Reis, Rui Oliveira, and João M L Dias. 2014. "Dynamic Metabolic Modelling of Volatile Fatty Acids Conversion to Polyhydroxyalkanoates by a Mixed Microbial Culture." *New Biotechnology* 31 (4): 335–44. doi:10.1016/j.nbt.2013.06.008.

Pratt, S., a. Werker, F. Morgan-Sagastume, and P. Lant. 2012. "Microaerophilic Conditions Support Elevated Mixed Culture Polyhydroxyalkanoate (PHA) Yields, but Result in Decreased PHA Production Rates." *Water Science & Technology* 65 (2): 243. doi:10.2166/wst.2012.086.

Serafim, Luísa S., Paulo C. Lemos, Rui Oliveira, and Maria a M Reis. 2004. "Optimization of Polyhydroxybutyrate Production by Mixed Cultures Submitted to Aerobic Dynamic Feeding Conditions." *Biotechnology and Bioengineering* 87 (2): 145–60. doi:10.1002/bit.20085.

Serafim, Luísa S, Paulo C Lemos, Cristiana Torres, Maria A M Reis, and Ana M Ramos. 2008. "The Influence of Process Parameters on the Characteristics of Polyhydroxyalkanoates Produced by Mixed Cultures." *Macromolecular Bioscience* 8 (4): 355–66. doi:10.1002/mabi.200700200.

Silva, Johanna a, Lorena M Tobella, José Becerra, Félix Godoy, and Miguel a Martínez. 2007.

"Biosynthesis of Poly-Beta-Hydroxyalkanoate by Brevundimonas Vesicularis LMG P-23615 and Sphingopyxis Macrogoltabida LMG 17324 Using Acid-Hydrolyzed Sawdust as Carbon Source." *Journal of Bioscience and Bioengineering* 103 (6): 542–46. doi:10.1263/jbb.103.542.

Tezuka, Y. 1969. "Cation-Dependent Flocculation in a Flavobacterium Species Predominant in Activated Sludge." *Applied Microbiology* 17 (2): 222–26.

Third, Katie a., Mark Newland, and Ralf Cord-Ruwisch. 2003. "The Effect of Dissolved Oxygen on PHB Accumulation in Activated Sludge Cultures." *Biotechnology and Bioengineering* 82 (2): 238–50. doi:10.1002/bit.10564.

Thomsen, Trine R, Linda L Blackall, Marilena Aquino de Muro, Jeppe L Nielsen, and Per H Nielsen. 2006. "Meganema Perideroedes Gen. Nov., Sp. Nov., a Filamentous Alphaproteobacterium from Activated Sludge." *International Journal of Systematic and Evolutionary Microbiology* 56 (Pt 8): 1865–68. doi:10.1099/ijs.0.02916-0.

Valentino, Francesco, Mario Beccari, Serena Fraraccio, Giulio Zanaroli, and Mauro Majone. 2014. "Feed Frequency in a Sequencing Batch Reactor Strongly Affects the Production of Polyhydroxyalkanoates (PHAs) from Volatile Fatty Acids." *New Biotechnology* 31 (4). Elsevier B.V.: 264–75. doi:10.1016/j.nbt.2013.10.006.

VANLOOSDRECHT, M, and M HENZE. 1999. "Maintenance, Endogeneous Respiration, Lysis, Decay and Predation." *Water Science and Technology* 39 (1): 107–17. doi:10.1016/S0273-1223(98)00780-X.

Vargas, Alejandro, Liliana Montaño, and Rodolfo Amaya. 2014. "Enhanced Polyhydroxyalkanoate Production from Organic Wastes via Process Control." *Bioresource Technology* 156 (March). Elsevier Ltd: 248–55. doi:10.1016/j.biortech.2014.01.045.

Villano, Marianna, Mario Beccari, Davide Dionisi, Silvia Lampis, Alfredo Miccheli, Giovanni Vallini, and Mauro Majone. 2010. "Effect of pH on the Production of Bacterial Polyhydroxyalkanoates by Mixed Cultures Enriched under Periodic Feeding." *Process Biochemistry* 45 (5). Elsevier Ltd: 714–23. doi:10.1016/j.procbio.2010.01.008.

4

METABOLIC MODELING OF THE SUB-STRATE COMPETITION AMONG MULTI-PLE VFAS FOR PHA PRODUCTION BY MIXED MICROBIAL CULTURES

4 METABOLIC MODELING OF THE SUB-STRATE COMPETITION AMONG MULTI-PLE VFAS FOR PHA PRODUCTION BY MIXED MICROBIAL CULTURES

SUMMARY: Mixed microbial cultures (MMCs) are an effective method for polyhydroxyalkanoates (PHA) production. There are several models established to describe the metabolism of this process. Substrate competition was commonly found in the uptake of multiple volatile fatty acids (VFAs), thus this behavior should be considered in process modeling. In this study, a metabolic model is developed to describe for the first time the substrate competition among four commonly used VFAs (acetate, propionate, butyrate and valerate) in PHA production by MMCs under high and low dissolved oxygen (DO) conditions. An inhibition parameter was introduced to describe the inhibition of butyrate and valerate on the uptake of acetate and propionate. The modeled results showed good agreement with experimental data at both DO levels within 95% confidence interval. This model enhanced the understanding of the metabolism of substrate uptake in PHA production, providing a successful approach to predict PHA composition and optimize the process efficiency when four competing VFAs are supplied.

Key words: Polyhydroxyalkanoates (PHAs), substrate competition, metabolic modeling, inhibition

Submitted as:

Wang Xiaofei, Gilda Carvalho, Maria A M Reis, Adrian Oehmen. 2017. “Metabolic modeling of the substrate competition among multiple VFAs for PHA production by mixed microbial cultures”. Journal of Biotechnology

4.1 Introduction

In order to describe and predict PHA production by MMCs, mathematical modeling has developed through various studies over the last years. The first metabolic models focused on PHB production from acetate (Beun et al. 2002; Dias et al. 2005), and quantified the energy requirement for both cell growth and maintenance through acetate uptake. The acetate-PHB models were further extended to P(HB-co-HV) production using the mixtures of acetate and propionate as carbon sources (Jiang et al. 2011; Dias et al. 2008). The production of intermediates acetyl-CoA and propionyl-CoA as well as the conversion between them were elaborated. Considering the complex composition in waste-based feedstocks, Pardelha et al. (2012) broadened the metabolic network to PHA production by multiple VFAs. In their work, VFAs with an odd and even number of carbon molecules followed different pathways to be converted to acetyl-CoA and propionyl-CoA, respectively. Tamis et al. (2014) reviewed the current models on PHA production, and proposed a generalized model to describe the feast-famine process of PHA accumulating cultures fed with mixed substrates, by dividing the substrate flux into different fractions for: PHB production, PHV production, and cell growth.

All of these models were established based on the assumption that all the VFAs were consumed simultaneously. However, in studies where the feedstock contained multiple VFAs, substrate competition was commonly observed and not all the VFAs were taken up at the same time. A significant preference of butyrate over acetate was found by Marang et al. (2013) when feeding acetate and butyrate simultaneously as carbon sources to a *Plasticicumulans acidivorans* dominated culture. Jiang et al. (2012) found that acetate and propionate consumption started to accelerate only after the depletion of butyrate, when feeding a selected culture with fermented paper mill effluent as carbon source. Chapter 3 showed that butyrate and valerate were the preferred substrates as compared to acetate and propionate regardless of the DO level applied in PHA production. The substrate preference is strongly related to the culture selected. As reported by Albuquerque et al. (2013), *Azoarcus* and *Thauera* had special preference for acetate and butyrate, while *Paracoccus* was adaptable to a wider range of VFAs.

This study aims to fill the gap in the understanding of VFA uptake kinetics in the PHA production process by MMCs, by developing a metabolic model to describe the substrate competition in these systems. Different DO levels are considered in the model calibration, in order to enhance the adjustability of this model to various operational conditions. This model can be applied to the feast phase of culture enrichment and the PHA accumulation step, for optimizing the process efficiency and predicting the PHA composition with the four common VFAs present in fermented wastes.

4.2 Materials and method

The detailed experimental setup and analytical techniques were described in Chapter 3. Briefly, two sequencing batch reactors (working volume 2L) were operated in parallel under the feast

and famine regime for PHA-accumulating cultures selection, SBR1 at high DO (3.47±1.12 mg/L) and SBR2 at low DO (0.86±0.50 mg/L) levels in the feast phase. Except for the aeration, other operational conditions were exactly the same in the two reactors at steady state. The feast phase was less than 1h in the 12h cycle. A mixture of VFAs (25% C-mol based acetic acid, propionic acid, butyric acid and valeric acid) was fed to both SBRs at the organic loading rate (OLR) of 50 Cmmol/L/d, together with other mineral nutrients medium. The C/N/P ratio was kept at 100:8:1. Ammonia was added 2h after the cycle started and consumed by the end of famine phase, to ensure no cell growth in the feast phase. The hydraulic retention time (HRT) was 1 day and the sludge retention time (SRT) was 4 days. The most abundant PHA accumulating bacteria selected in both cultures were *Plasticicumulans.*

Four batch tests were carried out to evaluate the substrate competition under different DO conditions in the PHA accumulation process. B1(HH) and B2(HL) were inoculated with the culture selected in SBR1 to accumulate PHAs under high DO (3.47±1.12 mg/L) and low DO (0.86±0.50 mg/L) conditions, respectively; B3(LH) and B4(LL) were inoculated with the culture selected in SBR2 to accumulate PHAs at the same high and low DO levels, respectively. The same composition of mixed VFAs and F/M ratio as applied in the culture selection phase were used for PHA accumulation in each batch test. A pulse-wise approach was conducted to supply substrates to the system. No ammonia was added in order to inhibit cell growth and maximize PHA production.

4.3 Metabolic model

4.3.1 Metabolism and reactions

The metabolic model constructed in this study was based on Pardelha et al. (2012), who described the uptake of VFAs in two pathways, with even and odd numbered carbons, which were converted to acetyl-CoA and acetyl-CoA + propionyl-CoA, respectively. However, their division of the pathways was not sufficient to describe the substrate competition, as observed in Chapter 3 that butyrate and valerate were taken up faster as compared to acetate and propionate. As Dias et al. (2006) pointed out, the conversion of butyrate and valerate could also proceed directly to the corresponding hydroxyacyl-CoA (hydroxybutyryl-CoA and hydroxyvaleryl-CoA, respectively). Hence, in this metabolic model, the uptake of each VFA, acetate, propionate, butyrate and valerate, was elaborated to its corresponding acyl-CoA (Figure 4-1). After the VFAs have been actively transported into cells, butyrate and valerate were assumed to be converted directly to PHB and PHV monomers (R3 and R4); meanwhile, acetate and propionate were converted to acetyl-CoA and propionyl-CoA (R1 and R2), respectively, through the same pathways as described by Dias et al. (2008). Part of the propionyl-CoA was decarboxylated to acetyl-CoA (R5) and the remainder was further converted to propionyl-CoA* (R10). The acetyl-CoA that didn't enter the TCA cycle through catabolism (R6), was converted to acetyl-CoA* (R9). Since no PH2MV was obtained in this study, the PHA composition followed the selective condensation (Dias et al. 2008): all propionyl-CoA* contributed to PHV production together

with part of the acetyl-CoA* (R11), where the rest of the acetyl–CoA* went to PHB. Besides catabolism, ATP was also generated through Oxidative Phosphorylation (R8) and consumed for maintenance (R7).

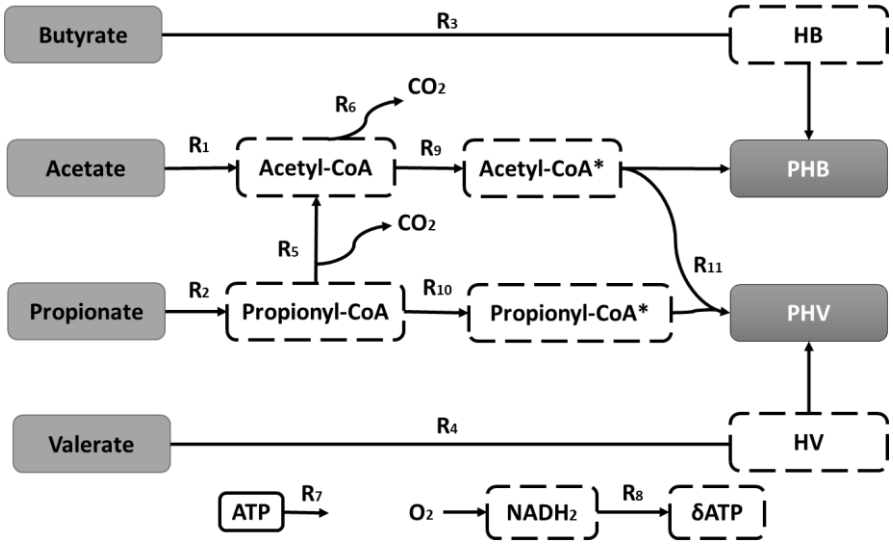

Figure 4-1 Metabolic model of PHAs production by a mixture of four VFAs

Table 4-1 Reactions for the metabolic model of PHA production from mixed VFAs (acetate, propionate, butyrate and valerate)

Reaction (carbon-mole basis)		stoichiometry
R_1	Acetate uptake	$CH_2O + ATP \rightarrow CHO_{0.5} + 0.5H_2O$
R_2	Propionate uptake	$CH_2O_{2/3} + 2/3\,ATP \rightarrow CH_{4/3}O_{1/3} + 1/3\,H_2O$
R_3	Butyrate uptake	$CH_2O_{0.5} + 0.5ATP \rightarrow CH_{1.5}O_{0.5} + 0.25NADH_2$
R_4	Valerate uptake	$CH_2O_{0.4} + 0.4ATP \rightarrow CH_{1.6}O_{0.4} + 0.2NADH_2$
R_5	Decarboxylation	$1.5CH_{4/3}O_{1/3} + H_2O \rightarrow CHO_{0.5} + 1.5NADH_2 + 0.5CO_2$
R_6	Catabolism	$CHO_{0.5} + 1.5H_2O \rightarrow CO_2 + 2NADH_2 + 0.5ATP$
R_7	Maintenance	$ATP \rightarrow m_{ATP}$
R_8	Oxidative Phosphorylation	$NADH_2 + 0.5O_2 \rightarrow H_2O + \delta ATP$
R_9	Acetyl-CoA* (PHB monomer) production	$CHO_{0.5} + 1/4NADH_2 \rightarrow CH_{1.5}O_{0.5}$
R_{10}	Propionyl-CoA* production	$CH_{4/3}O_{1/3} + 1/6NADH_2 \rightarrow CH_{5/3}O_{1/3}$
R_{11}	PHV monomer production	$0.4CH_{1.5}O_{0.5} + 0.6CH_{5/3}O_{1/3} \rightarrow CH_{1.6}O_{0.4}$
	PHB production	$R_{PHB} = R_9 + R_3 - 0.4R_{11}$
	PHV production	$R_{PHV} = R_{11} + R_4$

The reactions and stoichiometry of the metabolic model are presented in Table 4-1. There are overall 11 reactions, 5 intracellular intermediates (acetyl-CoA, propionyl-CoA, propionyl-CoA*, ATP and $NADH_2$), 5 input substrates (acetate, propionate, butyrate, valerate and O_2), and 3 end products (PHB monomer/ acetyl-CoA*, PHV monomer and CO_2). At steady state, the intermediates are balanced. The equations are shown in Eq (4.1)-(4.5).

Acetyl-CoA: $R_1 + R_5 - R_6 - R_9 = 0$ (Eq. 4.1)

Propionyl-CoA: $R_2 - 1.5R_5 - R_{10} = 0$ (Eq. 4.2)

Propionyl-CoA*: $R_{10} - 0.6R_{11} = 0$ (Eq. 4.3)

ATP: $-R_1 - 2/3R_2 - 0.5R_3 - 0.4R_4 + 0.5R_6 - R_7 + \delta R_8 = 0$ (Eq. 4.4)

NADH2: $0.25R_3 + 0.2R_4 + 1.5R_5 + 2R_6 - R_8 - 0.25R_9 - 1/6R_{10} = 0$ (Eq. 4.5)

In this work, the P/O ratio (δ) and the maintenance rate (R7) were fixed at predefined values (see Results). The decarboxylation rate (R5) was calculated through model calibration by fitting the experimental data of the inputs and outputs.

4.3.2 Kinetic model development

The observation of faster initial uptake rates of butyrate and valerate as compared to acetate and propionate indicated that the presence of butyrate and valerate inhibited the uptake of acetate and propionate (Chapter 3). In this metabolic model, an inhibition factor "K_i" was introduced to describe this effect. The maximum uptake rate of each individual VFA was expressed as a fraction (k_j) of the maximum total VFA uptake rate. Thus, after the "k_j" was calibrated, only one parameter, the uptake rate of total VFA was needed to be estimated for each test, instead of four uptake rates for the individual VFAs. The rates of substrate consumption and PHA production are listed in Table 4-2.

Table 4-2 Model kinetics

Dynamic process	Process rate
Acetate consumption	$k_{\_acet} * q_{\_VFA\_\max} * X * \frac{C_{\_acet}}{C_{\_acet} + K_{\_s\_VFA}} * \frac{K_{\_i}}{C_{\_but} + C_{\_val} + K_{\_i}}$
Propionate consumption	$k_{\_prop} * q_{\_VFA\_\max} * X * \frac{C_{\_prop}}{C_{\_prop} + K_{\_s\_VFA}} * \frac{K_{\_i}}{C_{\_but} + C_{\_val} + K_{\_i}}$
Butyrate consumption	$k_{\_but} * q_{\_VFA\_\max} * X * \frac{C_{\_but}}{C_{\_but} + K_{\_s\_VFA}}$

Valerate consumption	$k_{\_val} * q_{\_VFA\_\max} * X * \frac{C_{\_val}}{C_{\_val} + K_{\_s\_VFA}}$
PHB production	$0.7 * k_{\_acet} * q_{\_VFA\_\max} * X * \frac{C_{\_acet}}{C_{\_acet} + K_{\_s\_VFA}} * \frac{K_{\_i}}{C_{\_but} + C_{\_val} + K_{\_i}}$ $-\frac{13}{15} * k_{\_prop} * q_{\_VFA\_\max} * X * \frac{C_{\_prop}}{C_{\_prop} + K_{\_s\_VFA}} * \frac{K_{\_i}}{C_{\_but} + C_{\_val} + K_{\_i}}$ $+k_{\_but} * q_{\_VFA\_\max} * X * \frac{C_{\_but}}{C_{\_but} + K_{\_s\_VFA}} + \frac{13}{5} * q_{\_decxl} * X - 0.2 * q_{\_mATP} * X$
PHV production	$\frac{5}{3} * k_{\_prop} * q_{\_VFA\_\max} * X * \frac{C_{\_prop}}{C_{\_prop} + K_{\_s\_VFA}} * \frac{K_{\_i}}{C_{\_but} + C_{\_val} + K_{\_i}}$ $+k_{\_val} * q_{\_VFA\_\max} * X * \frac{C_{\_val}}{C_{\_val} + K_{\_s\_VFA}} - 2.5 * q_{\_decxl} * X$

4.3.3 Model calibration and evaluation

Aquasim V2.0 (Reichert 1998) was used to simulate the metabolic model. Parameter estimation was conducted through minimizing the chi-squared criterion χ^2 (weighted least squares) between the measured and simulated results. The experimental data of the two cultures, selected under both high-feast DO and low-feast DO in the two SBRs, were applied for model calibration, and the results of the four PHA accumulating batch tests were used for model validation.

The agreement between modeled results and experimental data was assessed by the square of the Pearson product moment correlation coefficient (R^2). The confidence bound Δy_{CI} was determined by the standard error of the y estimate (Se_y) within 95% confidence interval with the t-student distribution ($t_{\alpha,y}$) (Eq. 4.6).

$$\Delta y_{CI} = \frac{t_{\alpha,y} \cdot Se_y}{\sqrt{N}} \qquad \text{(Eq. 4.6)}$$

Sensitivity analysis using the absolute-relative sensitivity function (Eq. 4.7) (Reichert 1998) was performed to investigate the most determinant parameters of the model construction. The absolute change in each component (y) is measured for a 100% change of each estimated parameter (p).

$$\delta_{y,p}^{a,r} = p\frac{\partial y}{\partial p} \quad \text{(Eq. 4.7)}$$

4.4 Results and Discussion

4.4.1 Kinetic parameter estimation

The half-saturation constant of VFAs ($K_{\_s\_VFA}$), the efficiency of oxidative phosphorylation (P/O ratio, δ), and maintenance coefficient on ATP (m_{ATP}), were pre-defined as constants in this model simulation. Differences in the composition of MMCs and the variation of substrates can both impact the PHA production kinetics (Pardelha et al. 2014; Dias et al. 2008). The $K_{\_s\_VFA}$ value in this study was adopted from the work of Marang et al. (2015) as 0.3 Cmmol/L, since the culture selected by them was also dominated by the same microorganism, *Plasticicumulans acidivorans*. The values of m_{ATP} and P/O ratio applied in previous published works are listed in Table 4-3. Both parameters were either assumed or estimated by models. When feeding mixed VFAs, the P/O ratio was comparatively lower than the maximum value (3 molATP/mol$NADH_2$) obtained from acetate metabolism (Dias et al. 2008). The P/O ratio in this model was predefined as the most commonly used value of 2 molATP/mol$NADH_2$. As lower PHA production yield and maximum PHA storage were obtained in B2 (accumulating PHAs under low DO conditions using the culture selected at high feast DO), higher ATP requirements for maintenance were assumed (Chapter 3). Hence, a post- famine experiment was designed to calculate the m_{ATP} of each batch test (B1, B2, B3 and B4) by the consumption of PHA, where no external carbon sources were available. All the PHA consumed was assumed to be used for cell maintenance when no growth occurred under nutrient limiting conditions. Details of the experimental design and calculations are shown in Appendix B. m_{ATP} calculated from B1 (0.019 molATP/Cmol/h), B3 (0.010 molATP/Cmol/h) and B4 (0.005 molATP/Cmol/h) were similar. For simplification, an average value of 0.011±0.007 molATP/Cmol/h was applied when modeling with the data from these batch tests. This average m_{ATP} was also implemented to model the feast phase of SBR1 and SBR2, since both selection reactors were operated under the same conditions as B1 and B4, respectively. This value is close to the m_{ATP} estimated for heterotrophs 0.015 ± 0.005 molATP/Cmol/h (Beun et al. 2000a), and denitrifiers 0.02 ± 0.01 molATP/Cmol/h (Beun et al. 2000b) in PHA production processes, and also similar to the fixed value normally applied in other studies on metabolic modeling (0.02 ± 0.01 molATP/Cmol/h) (Dias et al. 2008; Pardelha et al. 2014; Dias et al. 2005). However, a comparatively higher value of m_{ATP} (0.075 molATP/Cmol/h) was obtained from B2, probably due to the high stress of low DO level on the maintenance of the culture selected in SBR1 (high DO in the feast phase) as discussed in Chapter 3. Hence, B2 was modeled using this higher mATP value.

As no cell growth occurred in the feast phase of culture selection and PHA accumulation batches, the constant biomass concentration (X) of each data set was determined by experimental measurements.

Table 4-3 m_{ATP} and P/O ratio from other models for PHA production by MMCs (values with * were estimated by models, without * were predefined.)

	m_{ATP} (molATP/Cmol/h)	P/O ratio (δ) (molATP/mol$NADH_2$)
Beun et al. (2000a)	0.015*	1.6*
Beun et al. (2000b)	0.02*	2*
Dias et al. (2005)	0.02	3*
Dias et al. (2008)	0.02	0.94-2.94*
Johnson et al. (2009)	0-0.12*	2
Pardelha et al. (2014)	0.02	0.63-1.7*
Jiang et al. (2011)	0.013-0.227*	1.7
Marang et al. (2015)	0.001	2
This work	0.011 for B1, B3, B4 and SBRs 0.075 for B2	2

Table 4-4 Kinetic parameter estimation

Parameter estimation by model calibration			
Decarboxylation rate	$q_{\_decxl}$	0.04±0.00*	Cmol/Cmol/h
inhibition factor	$K_{\_i}$	1.80±0.04*	Cmmol/L
coefficient of maximum acetate uptake rate per maximum total VFA uptake rate	$k_{\_acet}$	0.34±0.03*	Cmol/Cmol
coefficient of maximum propionate uptake rate per maximum total VFA uptake rate	$k_{\_prop}$	0.34±0.04*	Cmol/Cmol
coefficient of maximum butyrate uptake rate per maximum total VFA uptake rate	$k_{\_but}$	0.32±0.02*	Cmol/Cmol
coefficient of maximum valerate uptake rate per maximum total VFA uptake rate	$k_{\_val}$	0.29±0.01*	Cmol/Cmol
Maximum total VFA uptake rate estimated			
SBR1 (culture selection under high feast DO)	$q_{\_VFA\_max}$	1.00±0.08	Cmol/Cmol/h
SBR2 (culture selection under low feast DO)		1.00±0.05	Cmol/Cmol/h
B1 (culture from SBR1, with high DO)		1.51±0.05	Cmol/Cmol/h
B2 (culture from SBR1, with low DO)		0.82±0.03	Cmol/Cmol/h
B3 (culture from SBR2, with high DO)		1.96±0.07	Cmol/Cmol/h
B4 (culture from SBR2, with low DO)		1.08±0.05	Cmol/Cmol/h

* The value of each parameter is the average estimated by SBR1 and SBR2

The decarboxylation rate ($q_{\_decxl}$), the inhibition factor ($K_{\_i}$), and the coefficient ($k_{\_j}$) of the maximum individual VFA uptake rate per maximum total VFA uptake rate were estimated by calibrating the model using the data from SBR1 and SBR2 (Table 4-4). In order to minimize the impact of DO concentrations on the model calibration, the average values of these parameters were applied for the model validation by applying the data from 4 accumulation batches. Since the VFA uptake kinetics was largely dependent on the microbial communities enriched and the operational conditions (Pardelha et al. 2014), the maximum total VFA uptake rate ($q_{\_VFA\_max}$) of each data set was estimated separately (Table 4-4). Even though similar VFA uptake rates were

achieved in the selection step at different DO levels (SBR1 and SBR2), high DO was preferable to accumulate PHA at higher rates by both cultures selected (B1 and B3).

4.4.2 Modeling results

Figure 4-2 shows the measured data and the respective predictions of the calibrated model for culture selection (SBR1 and SBR2) at different DO levels in the feast phase, and for the PHA accumulation batches under different DO conditions of each culture selected (B1, B2, B3 and B4). It can be observed that this metabolic model can accurately simulate the substrate competition and PHA production performance for both culture selection and PHA accumulation stages. The inhibition factor $K_{\_i}$ was able to describe the initial inhibition of acetate and propionate when butyrate and valerate present. Even though the maximum total VFA uptake varied due to the population dynamics and the different DO conditions applied, the coefficient $k_{\_j}$ for the uptake of each individual VFA could be maintained as constant for satisfactory model prediction.

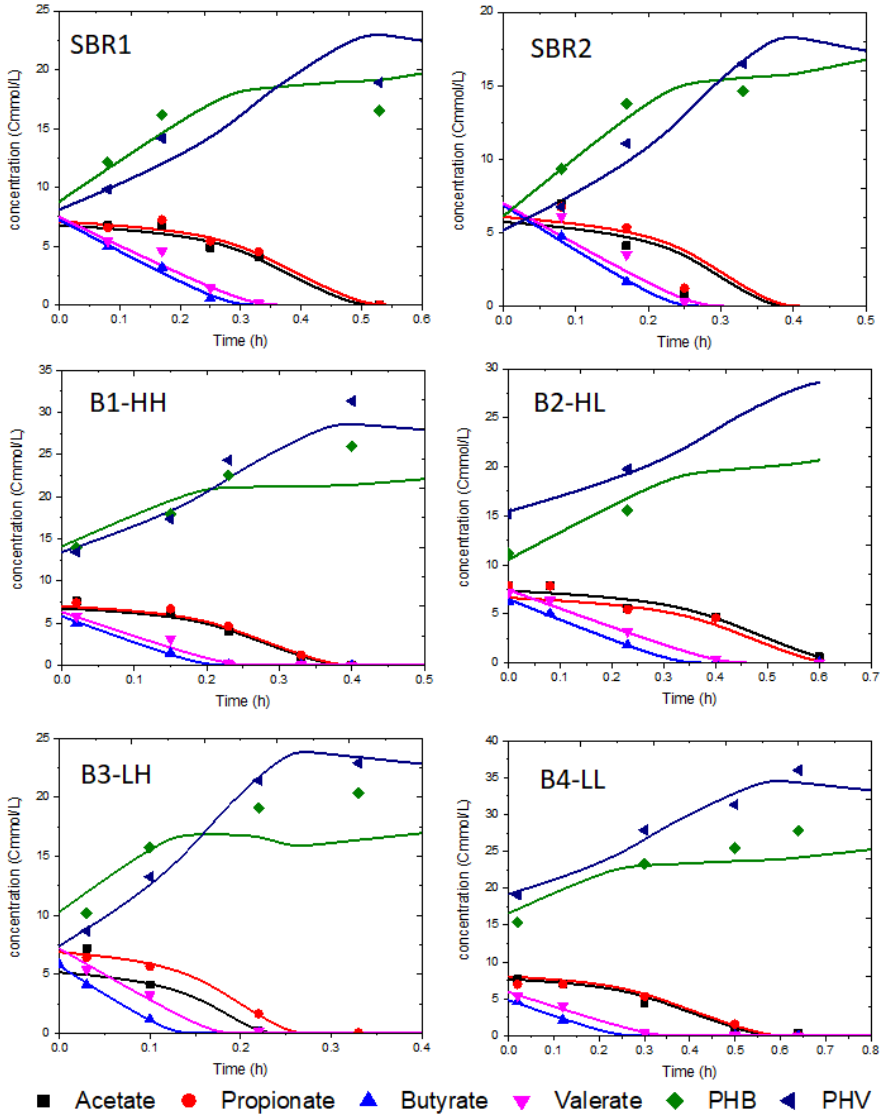

Figure 4-2 Model calibration results from SBR1 and SBR2, and model validation results from batch tests (B1, B2, B3 and B4), with experimental results in dots and simulation results in lines with corresponding colors.

The correlations of the modeled results with the measured data for each VFA taken up and PHA produced are evaluated individually (Figure 4-3). The majority of data points were within the 95% confidence interval, indicating the good agreement between the model predictions and the experimental measurements. The R^2 values close to 1 showed that the model was able to describe consistently all the processes involved in the experiments.

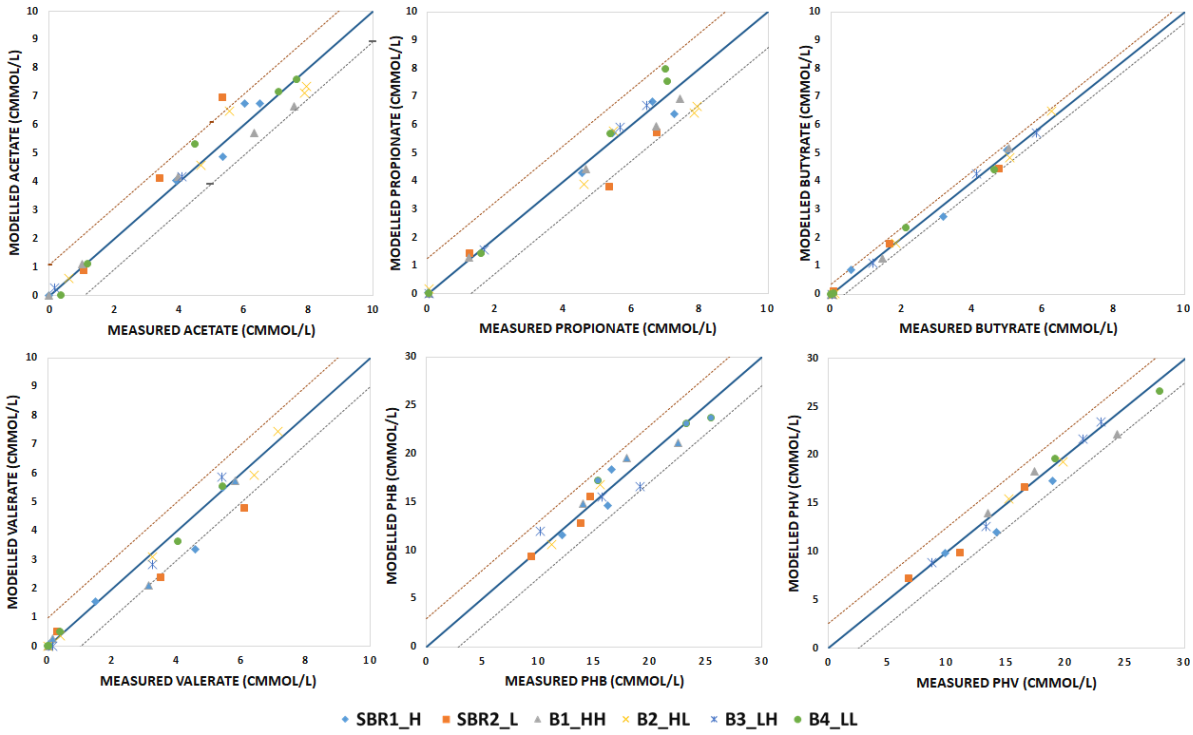

Figure 4-3 Correlation between modeled results and measured data. Full symbols represent the results of each data set applied. Dashed lines are the 95% confidence bounds.

4.4.3 Sensitivity evaluation

The absolute-relative sensitivity of all parameters, both predefined and estimated from the model, were assessed for the different processes: acetate uptake, propionate uptake, butyrate uptake, valerate uptake, PHB production, and PHV production. For example, in Figure 4-4-A, the sensitivity of $q_{\_VFA\_max}$ from each data set (SBR1, SBR2, B1, B2, B3 and B4) to the acetate uptake process was calculated and then combined together. The combined value indicated the total sensitivity of $q_{\_VFA\_max}$ to the acetate uptake process from all data sets. The total sensitivity of each parameter to the acetate uptake process was calculated by the same method, then ranked in Figure 4-4-A. The ranks of parameter sensitivity in other processes following the same calculation are shown in Figure 4-4-B to F, for propionate uptake, butyrate uptake, valerate uptake, PHB production and PHV production, respectively.

The maximum VFA uptake rate ($q_{\_VFA\_max}$) was the most sensitive among all the kinetic parameters, with the first rank in all the processes, except for PHB production (ranked second). Thus, it was essential to estimate this parameter in each data set due to the variation along the different batch tests. The coefficient of maximum individual VFA uptake rate on maximum total VFA uptake rate ($k_{\_j}$) was the second most sensitive parameter in each VFA uptake process. In butyrate (Figure 4-4-C) and valerate (Figure 4-4-D) uptake processes, $k_{\_but}$ and $k_{\_val}$ were equally sensitive to $q_{\_VFA\_max}$, respectively, since they were the only important factors other than $q_{\_VFA\_max}$ to determine the substrate uptake rates. However, the uptake of acetate (Figure 4-4-A)

and propionate (Figure 4-4-B) were sensitive to more parameters besides their own coefficients $k_{\_acet}$ and $k_{\_prop}$, such as $k_{\_val}$, $k_{\_i}$ and $k_{\_but}$. This was due to the inhibition effect on acetate and propionate uptake rates when butyrate and valerate present. The $K_{\_s\_VFA}$ had minor sensitivity in all processes. This predefined constant was applicable for the kinetic modeling but does not necessarily reflect the true value of this parameter. As also stated by Johnson et al. (2009), a small change in the $K_{\_s\_VFA}$ would not impact the outcome of the model simulation, which is consistent with the results of Figure 4. PHV production was more sensitive to $k_{\_prop}$ and $k_{\_val}$ than the acetate and butyrate uptake coefficients, since propionate and valerate were the only VFAs determining PHV production. Meanwhile, not only were the uptake coefficients of direct PHB contributors like acetate ($k_{\_acet}$) and butyrate ($k_{\_but}$) sensitive to PHB production, but also $k_{\_prop}$. This is because the propionate uptake determined the amount of acetyl-CoA that was subtracted from PHB production to form PHV in the selective condensation of PHA as described in section 4.3.1.

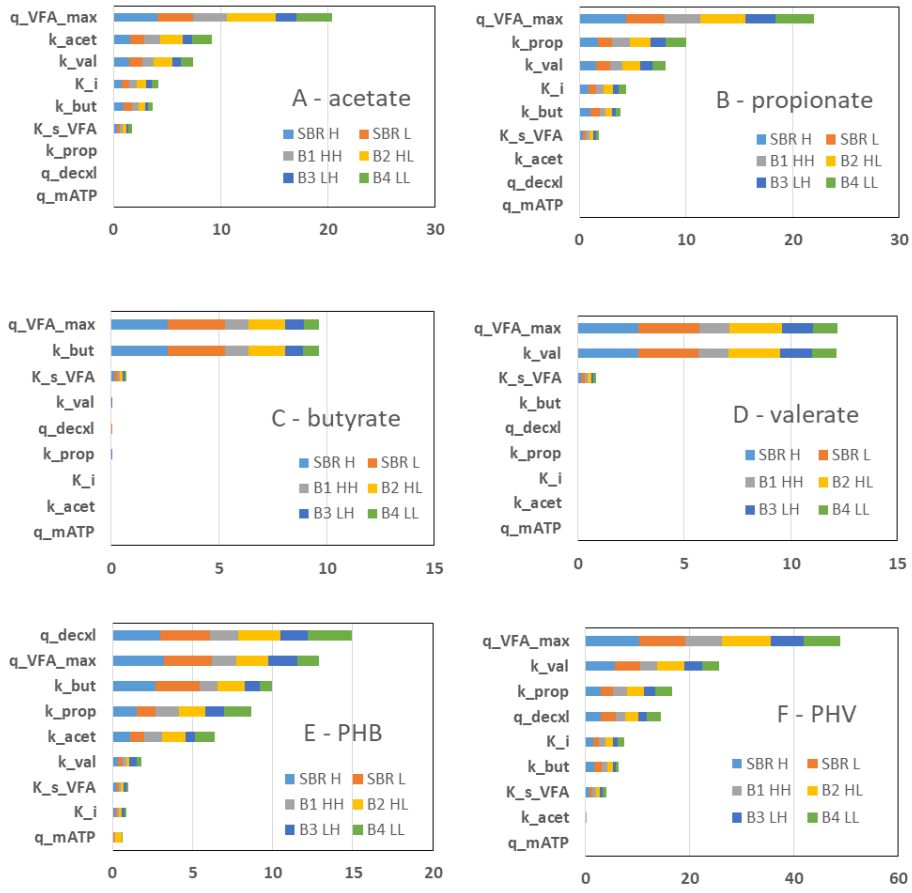

Figure 4-4 Rank of the sum-up of absolute-relative sensitivity values av (|SensAR|) in Cmmol/L of each parameter in each process: A: acetate uptake; B: propionate uptake; C: butyrate uptake; D: valerate uptake; E: PHB production, and F: PHV production.

The decarboxylation rate ($q_{\_decxl}$) had negligible sensitivity towards VFA uptake but significantly impacted the PHA production. Even though $q_{\_decxl}$ ranked differently for PHB and PHV pro-

duction, the absolute values were similar. With the value as low as 0.04, almost no decarboxylation occurred in this model. The maintenance rate ($q_{\_mATP}$) was the least sensitive parameter in all processes. Compared to the energy transfer of substrate uptake, the ATP requirement for maintenance seemed to be negligible. This may explain why in other studies mATP was often predefined at a fixed value.

The different DO levels applied only had an impact on the maximum total VFA uptake rates, but not on the $k_{\_j}$ or other parameters estimated. This model was able to predict not only the PHA composition, but also offered an approach to describe the substrate competition among the four commonly used VFAs that had never been discussed in metabolic modeling before. In future modelling endeavors, when a feedstock with different VFA composition is applied, $k_{\_j}$ may need to be adjusted for a proper fitting.

4.5 Conclusion

A robust metabolic model was developed in this study to describe the substrate competition of four VFAs (acetate, propionate, butyrate and valerate) in the PHA production process by mixed microbial cultures. This model was applicable to both the feast phase of culture selection and the PHA accumulation stage under different DO conditions. The stoichiometric and kinetic parameters determined from this model can be used to predict VFA consumption and PHA production where substrate competition occurs. However, it should be noted that cell growth was excluded from this model development due to ammonia deficiency in the feast phase. The substrate competition for simultaneous PHA and biomass production requires further study.

References

Albuquerque, Maria G E, Gilda Carvalho, Caroline Kragelund, Ana F Silva, Maria T Barreto Crespo, Maria A M Reis, and Per H Nielsen. 2013. "Link between Microbial Composition and Carbon Substrate-Uptake Preferences in a PHA-Storing Community." *The ISME Journal* (2013) 7,1 (1751–7362/13).

Beun, J. J., K. Dircks, M. C M Van Loosdrecht, and J. J. Heijnen. 2002. "Poly-B-Hydroxybutyrate Metabolism in Dynamically Fed Mixed Microbial Cultures." *Water Research* 36 (5): 1167–80. doi:10.1016/S0043-1354(01)00317-7.

Beun, J. J., F. Paletta, M. C M Van Loosdrecht, and J. J. Heijnen. 2000. "Stoichiometry and Kinetics of Poly-β-Hydroxybutyrate Metabolism in Aerobic, Slow Growing, Activated Sludge Cultures." *Biotechnology and Bioengineering* 67 (4): 379–89. doi:10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2.

Beun, J J, E V Verhoef, M C M Van Loosdrecht, and J J Heijnen. 2000. "Stoichiometry and Kinetics of Poly-Beta-Hydroxybutyrate Metabolism under Denitrifying Conditions in Activated Sludge Cultures." *Biotechnology and Bioengineering* 68 (5): 496–507. doi:10.1002/(SICI)1097-0290(20000220)67:4<379::AID-BIT1>3.0.CO;2-2.

Dias, João M.L., Luísa S. Serafim, Paulo C. Lemos, Maria a.M. Reis, and Rui Oliveira. 2005. "Mathematical Modelling of a Mixed Culture Cultivation Process for the Production of Polyhydroxybutyrate." *Biotechnology and Bioengineering* 92 (2): 209–22. doi:10.1002/bit.20598.

Dias, João M L, Paulo C. Lemos, Luísa S. Serafim, Cristina Oliveira, Marta Eiroa, Maria G E Albuquerque, Ana M. Ramos, Rui Oliveira, and Maria a M Reis. 2006. "Recent Advances in Polyhydroxyalkanoate Production by Mixed Aerobic Cultures: From the Substrate to the Final Product." *Macromolecular Bioscience* 6 (11): 885–906. doi:10.1002/mabi.200600112.

Dias, João M L, Adrian Oehmen, Luísa S Serafim, Paulo C Lemos, Maria A M Reis, and Rui Oliveira. 2008. "Metabolic Modelling of Polyhydroxyalkanoate Copolymers Production by Mixed Microbial Cultures." *BMC Systems Biology* 2 (1): 59. doi:10.1186/1752-0509-2-59.

Jiang, Yang, Marit Hebly, Robbert Kleerebezem, Gerard Muyzer, and Mark C M van Loosdrecht. 2011. "Metabolic Modeling of Mixed Substrate Uptake for Polyhydroxyalkanoate (PHA) Production." *Water Research* 45 (3): 1309–21. doi:10.1016/j.watres.2010.10.009.

Jiang, Yang, Leonie Marang, Jelmer Tamis, Mark C M van Loosdrecht, Henk Dijkman, and Robbert Kleerebezem. 2012. "Waste to Resource: Converting Paper Mill Wastewater to Bioplastic." *Water Research* 46 (17). Elsevier Ltd: 5517–30. doi:10.1016/j.watres.2012.07.028.

Johnson, Katja, Robbert Kleerebezem, and Mark C M van Loosdrecht. 2009. "Model-Based Data Evaluation of Polyhydroxybutyrate Producing Mixed Microbial Cultures in Aerobic Sequencing Batch and Fed-Batch Reactors." *Biotechnology and Bioengineering* 104 (1): 50–67. doi:10.1002/bit.22380.

Marang, Leonie, Yang Jiang, Mark C M van Loosdrecht, and Robbert Kleerebezem. 2013. "Butyrate as Preferred Substrate for Polyhydroxybutyrate Production." *Bioresource Technology* 142 (August). Elsevier Ltd: 232–39. doi:10.1016/j.biortech.2013.05.031.

Marang, Leonie, Mark C M van Loosdrecht, and Robbert Kleerebezem. 2015. "Modeling the

Competition between PHA-Producing and Non-PHA-Producing Bacteria in Feast-Famine SBR and Staged CSTR Systems." *Biotechnology and Bioengineering* 112 (12): 2475–84. doi:10.1002/bit.25674.

Pardelha, Filipa, Maria G E Albuquerque, Maria a M Reis, João M L Dias, and Rui Oliveira. 2012. "Flux Balance Analysis of Mixed Microbial Cultures: Application to the Production of Polyhydroxyalkanoates from Complex Mixtures of Volatile Fatty Acids." *Journal of Biotechnology* 162 (2–3). Elsevier B.V.: 336–45. doi:10.1016/j.jbiotec.2012.08.017.

Pardelha, Filipa, Maria G E Albuquerque, Maria a M Reis, Rui Oliveira, and João M L Dias. 2014. "Dynamic Metabolic Modelling of Volatile Fatty Acids Conversion to Polyhydroxyalkanoates by a Mixed Microbial Culture." *New Biotechnology* 31 (4): 335–44. doi:10.1016/j.nbt.2013.06.008.

Reichert, Peter. 1998. *Computer Program for the Identi Cation and Simulation of Aquatic Systems*.

Tamis, Jelmer, Leonie Marang, Yang Jiang, Mark C.M. van Loosdrecht, and Robbert Kleerebezem. 2014. "Modeling PHA-Producing Microbial Enrichment Cultures—towards a Generalized Model with Predictive Power." *New Biotechnology* 31 (4). Elsevier B.V.: 324–34. doi:10.1016/j.nbt.2013.11.007.

5

THE IMPACT OF DO ON PHA ACCUMULATING CULTURE SELECTION AND THE QUANTIFICATION OF DO AFFINITY ON VFA UPTAKE

SUMMARY: As discussed in previous studies, aeration plays an important role in the PHA production by mixed microbial cultures, in terms of the energy cost reduction and process optimization. In this study, the impact of oxygen on both feast and famine phases in the culture selection stage was investigated, and the affinity to DO of different cultures selected on the uptake of each of four VFAs (acetate, propionate, butyrate and valerate) was for the first time quantified. At a non-limiting DO level (3.79±0.65 mg/L), a *Paracoccus* dominating culture was selected, while the PHA accumulating bacteria failed to be enriched at a limited DO level (0.48±0.29 mg/L) due to settling problems, likely due to the excess production of exopolysaccharides (EPS). By characterizing the microbial community dynamics under the unlimited DO conditions, it was found that *Paracoccus* was the dominating population (>50%) in the selected cultures, and substrate preference on butyrate and valerate was correlated with the abundance of *Plasticicumulans* during culture selection.

Key words: DO affinity; VFA uptake, substrate preference, *Plasticicumulans*

5.1 Introduction

Aeration is the key factor of the operational costs in PHA production process by mixed microbial cultures (MMCs), especially in the culture enrichment step. As stated in chapter 3, under both the high (3.47 ± 1.12 mg/L) and low (0.86 ± 0.50 mg/L) DO conditions applied in the feast phase, MMCs containing mostly the same populations but with different relative abundance, with similar PHA production efficiency. Another study showed that by reducing the air input to the famine phase of a PHA accumulating process by aerobic granules, the VFA consumption and PHA accumulation rates in the feast phase were accelerated, resulting in the decreased feast duration, with the same amounts of PHA accumulated and COD removed (Vjayan and Vadivelu 2017). However, in their study, by reducing air flow from 1 vvm to 0.5 and 0.25 vvm, the DO concentration was not influenced, which means the saturated DO level was still maintained in the famine phase. Coats et al. (2016) studied the impact of aeration strategy (K_La at 4, 8, 12 and 20 hr^{-1}) on the enrichment of PHA accumulating cultures, showing that there was no statistical difference in the average PHA yield, although the VFA conversion to PHA was more efficient under higher aeration states. In their work, diverse MMCs were selected under different aeration conditions, with the common dominant population of *Meganema* by feeding fermented dairy manure as the carbon sources.

It is worth noticing that no significant impact of aeration on the microbial composition of the selected MMC was observed in these studies. Even though the DO concentrations, K_La, or aeration rates were reduced, none of the MMC enrichments were operated under high selection pressure of DO limitation. As reported by previous studies, PHA accumulation could occur in the anoxic phase of nitrogen removal process in WWTP (D Dionisi et al. 2004; Bengtsson et al. 2017; Anterrieu et al. 2014), which indicated that certain microorganisms were able to accumulate PHAs under DO limited conditions. It was hypothesized that different PHA storing bacteria had different affinity to DO. By supplying limited DO concentrations, different microorganisms may be selected compared to the unlimited aeration condition. Hence, one aim of this work, is to study the MMC enrichments under both DO-limited (0.48±0.29 mg/L) and DO-rich (3.79±0.65 mg/L) conditions, through the entire feast and famine phases in each SBR.

As hypothesized in chapter 3, the consumption of longer chain VFAs has likely higher affinity for DO as compared to the shorter chain VFAs. Especially when using the MMCs selected at lower feast DO levels to accumulate PHA, the uptake rates of butyrate and valerate were not as significantly impacted as acetate and propionate. Another aim of this study is to verify this hypothesis more directly, by quantifying the DO affinity of each VFA using the MMCs selected under the two DO conditions. It was anticipated to observe higher DO affinity on butyrate and valerate uptakes than acetate and propionate by using both cultures selected. Since the microbial community enriched contributes to the DO affinity for carbon substrates, the DO affinity of butyrate and valerate should be even higher when using cultures selected at low DO levels as compared to those selected at high DO levels.

5.2 Materials and methods

5.2.1 MMC selections under strict DO control in SBRs

Two SBRs (2L), inoculated with activated sludge from the Chelas WWTP in Lisbon, Portugal, were operated in parallel for MMC selection under different DO conditions (SBR1 with limited DO at 0.48±0.29 mg/L; SBR2 with unlimited DO at 3.79±0.65 mg/L). The DO concentrations were kept constant through the entire cycle in both feast and famine phases. The same software as mentioned in chapter 3 was also applied in this study for acquiring pH and DO data and controlling the on/off of the acid dosing pump and air input valves. Since the aeration requirements for VFA uptake in the feast phase and cell growth in the famine phase are different, the DO levels could not be maintained constant with acceptable variations by only using the on/off valves, especially at low DO levels. Hence, an air flow controller was installed between the air pump and the valve of each SBR system. The air input for SBR1 (limited DO) was controlled at 1L/min in the feast phase, and 0.5L/min in the famine phase, while the air flow for SBR2 (unlimited DO) was kept at 4L/min in the feast phase, and 2L/min in the famine phase. Thus, by the cooperation of on/off valves and air flow controllers, the DO of both SBRs were maintained at the designated constant levels throughout the feast and famine phases.

Except for the different DO concentrations, other operational conditions were kept the same for both SBRs. To ensure a steady startup and mitigation of the washing out of the biomass, the initial sludge retention time (SRT) was set at 8 days then reduced to 4 days after the first week; the initial organic loading rate (OLR) was 12.5 Cmmol/L/d, and then increased to 50 Cmmol/L/d gradually in 4 weeks. The C/N/P ratio was kept the same at 100/7/1 along with the adjustment of OLR. The hydraulic retention time (HRT) was 1 day. pH was controlled between 7 and 8 by dosing HCl (0.5M). The temperature was kept at 20 ºC by a water bath. The aeration was supplied through ceramic diffusers and stirring was performed by overhead stirrers (at 200 rpm). The 12h working cycle automatically controlled by the software was composed of 5 periods: feeding (5 min), aeration (11h 10 min), purge (5 min), settling (30min), and withdrawal (10 min). The medium fed to the SBRs at the beginning of each cycle consisted of mixed VFAs (25% C-mol based acetic acid, propionic acid, butyric acid and valeric acid), mineral nutrients and thiourea (10 mg/L) for nitrification inhibition (details of the nutrients composition in chapter 3). NH4Cl was only added 2h after the start of the cycle to enhance the selective pressure for PHA accumulating communities by eliminating the cell growth in the feast phase (Oliveira et al. 2016).

5.2.2 Batch_DO: Batch tests for the quantification of DO affinity with each VFA

Seven batch tests were carried out over 4 days under different DO conditions (e.g. 1%, 3%, 5%, 10%, 25%, 40% and 60% DO saturation, exact values of the DO concentration are shown in section 5.3.2) to quantify the DO affinity by measuring the specific substrate uptake rate of each VFA. The mixture of VFAs (25% Cmol of acetate, propionate, butyrate and valerate, respectively) was fed as the carbon source to each batch with the initial concentration of 20 Cmmol/L. The working volume of each reactor was 500 ml. The biomass was purged from the selection

SBRs at the end of famine phase, then aerated for 2h before it was inoculated to the batch reactors. The temperature was maintained at 20 ºC by a water bath. pH was controlled at 7-8 by dosing HCl (1M). The agitation was kept at 180 rpm when operating at high DO level (> 30% DO saturation), and 100 rpm when operating at low DO level (< 30% DO saturation). The control and acquisition of the parameters, including DO, agitation, pH and temperature were accomplished by the New Brunswick™ BioFlo®/ CelliGen® 115 Bioreactor system from EPPENDORF.

The DO affinity during VFA uptake was indicated by the half-velocity constant (K_{DO}) from modified Michaelis-Menten kinetics (Eq.5.1). The higher K_{DO} means a lower affinity to DO.

$$q_{VFA} = q_{\max\_VFA} * \frac{C_s}{C_s + K_s} * \frac{C_{DO}}{C_{DO} + K_{DO}} \quad \text{(Eq.5.1)}$$

q_{VFA}: the specific VFA uptake rate

$q_{\max\_VFA}$: the maximum specific VFA uptake rate

C_s: VFA concentration

K_s: half velocity constant of VFA

C_{DO}: DO concentration

K_{DO}: half velocity constant of DO

Since the concentrations of VFAs were not limited, $\frac{C_s}{C_s + K_s}$ was assumed as 1. q_{max_VFA} and K_{DO} of each VFA were estimated (in Sigmaplot V12.5) by fitting the q_{VFA} obtained from the batch tests at each DO level (C_{DO}) to the modified Michaelis-Menten kinetics.

5.2.3 Analytical methods and parameter determination

The contents of VFAs (acetate, propionate, butyrate and valerate) were determined by high performance liquid chromatography (HPLC); while the concentrations of PHAs (PHB and PHV) were measured by gas chromatography (GC). Skalar San++ system was used for the measurements of ammonium and phosphate. Volatile suspended solids (VSS) were measured according to the standard methods (APHA 1999) to determine the cell dry weight. Nile Blue staining was applied for the observation of PHA granules and the morphology of microorganisms. The quantification of the abundance of each bacteria was implemented by high throughput DNA sequencing in the company DNASense Aps. More details regarding these analytical methods were explained in chapter 3.

VSS was composed by active biomass (X) and PHAs. The content of PHA was determined as %PHA = PHA/VSS*100 (g PHA/g VSS), and the content of active biomass was calculated as X = VSS-PHA (g). The standard biomass was assumed as $CH_{1.8}O_{0.5}N_{0.2}$, with a molecular weight of 24.6 g/Cmol. The specific VFA uptake rate (q_{VFA} in Cmol VFA/ Cmol X/h) or PHA

accumulation rate (q_{PHA} in Cmol PHA/ Cmol X/h) were calculated by the slope of VFA or PHA concentrations in the corresponding time period in the feast phase per Cmol of biomass. The yield of PHA production per substrate consumption was determined as $Y_{PHA/S} = q_{PHA}/ q_{VFA}$.

5.3 Results and discussion

5.3.1 MMC selection under high and low DO conditions

The DO concentration was strictly controlled at 0.48±0.29 mg/L and 3.79±0.65 mg/L in SBR1 and SBR2, respectively, in both feast and famine phases through the entire cycle, to investigate the impact of DO levels on the PHA accumulating culture selection. Figure 5-1 shows the performance of the two reactors along the operational time, displaying the changes of the feast and famine (F/F) ratio, VSS concentrations, and total amount of air input in each cycle, from day 28 when both reactors were operated under the SRT of 4d and organic loading rate of 50 Cmmol/L/d.

In SBR1 with low DO conditions, the culture selection was relatively stable from day 28 to day 80, with the F/F ratio 0.13±0.03 and the VSS concentration 2.29±0.08 g/L. The amount of air input of each cycle reduced from 112.75±7.89 L in day 30-60, to 81.67±10.82 L in day 60-80, indicating that less active bacteria with a lower oxygen requirement was selected over time. A typical operational cycle (on day 66) of the culture selection is shown in Figure 5-2. However, after day 80, the supernatant after settling became opaque, and the boundary between the supernatant and sludge blanket was unclear. The biomass sampled in the feast phase contained a jelly-like layer after centrifuging. It could be caused by the formation of exopolysaccharides (EPS), the extracellular carbon storage product that can be produced by various bacteria while accumulating intracellular PHAs (Pagliano et al. 2017). Filamentous bacteria (e.g. *Meganema*) was observed with a high abundance, which was often reported to be responsible for bulking in industrial wastewater treatment plants (Levantesi et al. 2004), also leading to the settling problems. The VSS concentrations decreased gradually due to the wash out of the biomass caused by the poor settling properties. As a consequence of the low biomass concentration, the F/F ratio increased significantly. Correspondingly, the total amount of air input increased, as the feast phase with higher energy requirement was extended. On day 130, the VSS concentration reduced to 0.88±0.02 g/L with the feast phase longer than 5h in the 12h cycle. According to Dionisi et al. (2007), the feast phase should be within 20% of the length of the entire cycle for the selection of the MMCs with good capacity of PHA storage. Thus, these results suggest that this DO concentration (0.48±0.29) mg/L in SBR1 might be too low for PHA accumulating culture selection in long term operation.

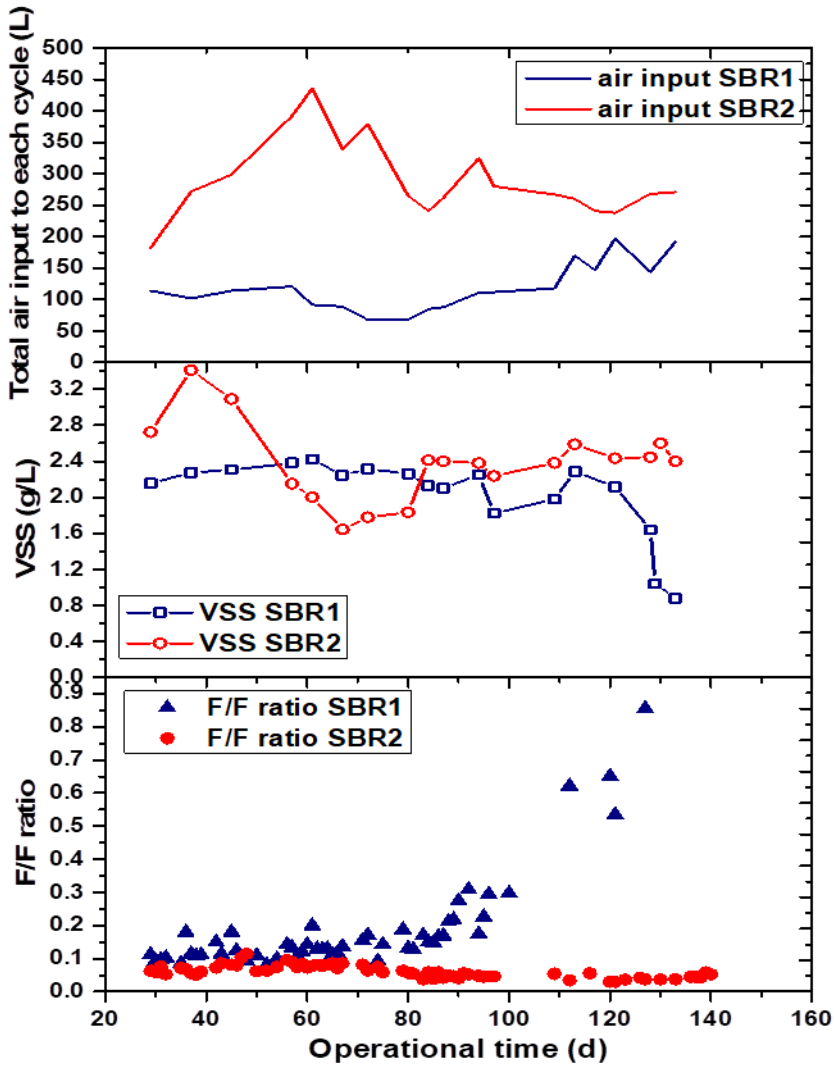

Figure 5-1 Feast/famine ratio, VSS, and the total air input over the time in SBR1 and SBR2 for culture selection.

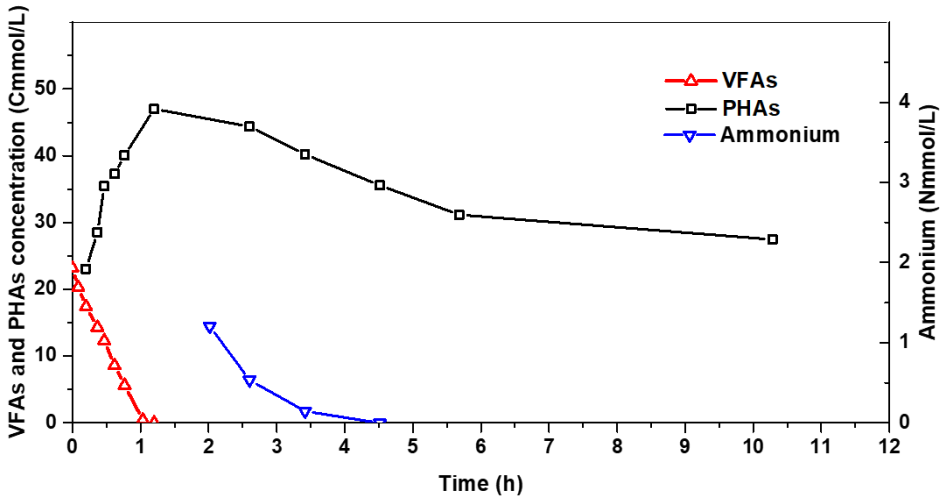

Figure 5-2 An operational cycle in SBR1 for culture selection under low DO (on day 66)

In SBR2, with high DO conditions, the F/F ratio increased gradually to 0.07±0.01 in the first 50 days. On day 62, part of the biomass was washed out due to system failure at the end of the cycle, and then recovered on the next day by putting back the purge. This accident impacted the amount of total air input and the VSS concentration but did not significantly affect the F/F ratio as shown in Figure 5-1. After day 80, the culture selection process in SBR2 achieved steady state, with the average VSS concentrations 2.35±0.21 g/L, the air input amount 265.30±25.36 L per cycle, and the F/F ratio 0.05±0.01.

Due to the failure of the culture selection stage under low DO conditions, the analysis on substrate competition was only made on the SBR2. Figure 5-3 depicts the substrate consumption during feast for three days along the culture selection at high DO levels. It is interesting to observe that the substrate competition appeared along with the evolution of the microbial communities in SBR2 with high DO concentrations. The entire MMC selecting process could be briefly divided into 3 stages according to the substrate preference. Stage1: day 1-40, the initial period of culture selection when no substrate competition was observed. The four VFAs were taken up simultaneously at similar rates (e.g. around 0.08 Cmol VFA/Cmol X/h on day 36 in Figure 5-3a). Stage 2: day 40 -120, when the MMC was mainly selected but not stabilized yet, the consumption of propionate was slightly slower than the other three VFAs (e.g. on day 92 in Figure 5-3b). Stage 3: day 120 -150, when steady PHA accumulating culture was selected, strong preference of butyrate and valerate over acetate and propionate was observed (e.g. on day 135 in Figure 5-3c). The specific VFA uptake rates in the 3 days are listed in Table 5-1. The uptake rates of the total VFAs increased along with the selection process through the 3 stages. Since the operational conditions were kept the same, the preference of the substrate could be highly related to the composition of the microbial community in the MMC.

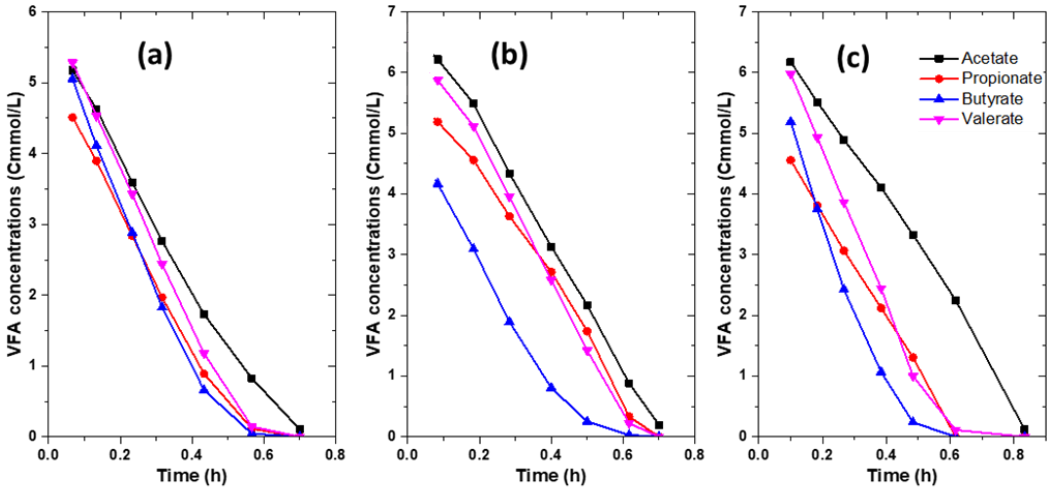

Figure 5-3 VFA consumption in the feast phase of SBR2 under high DO conditions on day 36 (a), day 92 (b), and day 135 (c)

Table 5-1 The specific VFA uptake rates on day 36, 92, and 135 in the feast phase of SBR2 under high DO conditions

	specific VFA uptake rates in SBR2 (Cmol VFA/Cmol X/h)		
	day 36	day 92	day 135
Acetate	0.07±0.001	0.17±0.003	0.12±0.002
Propionate	0.08±0.001	0.14±0.004	0.14±0.002
Butyrate	0.09±0.003	0.18±0.001	0.23±0.016
Valerate	0.08±0.001	0.18±0.004	0.21±0.004
Total VFAs	0.32±0.005	0.64±0.007	0.67±0.017

High throughput amplicon sequencing of the 16S rRNA gene was applied to characterize the microbial populations of samples collected in stage 2 (on day 112) and stage 3 (on day 144), respectively. The most abundant populations of the two MMCs are listed in Table 5-2. Around 74% of the bacteria enriched in each culture were reported as PHA storers by previous studies. *Paracoccus* was the dominant community in both MMCs, comprising more than 50% of the total population. This bacteria was commonly found as a PHA storer in the culture selection process for PHA production (Queirós, Rossetti, and Serafim 2014; Carvalho et al. 2014), with the ability to take up a broad range of VFAs including acetate, propionate, butyrate and valerate (Albuquerque et al. 2013). *Aminobacter* has been identified in biological nutrient removal processes as a denitrifier with the ability to accumulate PHB in the cells (Kondo et al. 2009; Urakami et al. 1992). This microorganism was the second dominant population on day 112 (11.1%) and the third on day 144 (5.1%). However, few studies can be found on the culture en-

richment for PHA production reporting the selection of *Aminobacter*. *Plasticicumulans* showed relatively high abundance in both cultures, with the percentage of 7.7% on day 112 and 18.2% on day 144. This microorganism was the most abundant in the selected culture in previous experiments (chapter 3), and was reported firstly to be enriched in acetate fed cultures with good adaptability to take up other VFAs for PHA production (Johnson, Kleerebezem, and van Loosdrecht 2009; Tamis et al. 2014). *Meganema*, a PHA accumulating bacterium that had been found in various studies for PHA production (Davide Dionisi et al. 2005; Coats, Watson, and Brinkman 2016), constituted a relatively lower fraction in both MMCs.

Table 5-2 The 6 most abundant bacteria identified by 16S rRNA gene amplicon sequencing of the cultures enriched in high DO SBR2 on day 112 and 144 in the culture selection step.

	Day 112			Day 144		
	Phylum	Genus/OTU	%	Phylum	Genus/OTU	%
1	Alphaproteobacteria	***Paracocccus***	52.2	Alphaproteobacteria	***Paracocccus***	51.2
2	Alphaproteobacteria	***Aminobacter***	11.1	Gammaproteobacteria	***Plasticicumulans***	18.2
3	Bacteroidetes	OTU_21	10.4	Alphaproteobacteria	***Aminobacter***	5.1
4	Gammaproteobacteria	***Plasticicumulans***	7.7	Alphaproteobacteria	***Meganema***	4.5
5	Alphaproteobacteria	***Meganema***	2.4	Bacteroidetes	*Flavobacterium*	2.8
6	Bacteroidetes	*Flavobacterium*	0.7	Alphaproteobacteria	OTU_17	2.2
	PHA storers in total (**in Bold**)		**73.4**	PHA storers in total (**in Bold**)		**74**

The preference on VFAs of different bacteria was reported by various studies. The cultures consisting of *Paracoccus* (50%), *Azoarcus* (26%) and *Thauera* (8%) selected by Albuquerque et al. (2013) showed a strong preference on butyrate with the highest consumption rate. Butyrate and valerate were the preferable substrates for the majority of the population when fed together with acetate and propionate. When butyrate and valerate were depleted, *Azoarcus* and *Thauera* started to take up propionate, while acetate consumption increased due to *Azoarcus* and *Paracoccus*. In the studies that enriched *Plasticicumulans*-dominated cultures, a clear preference of the longer chain VFAs was also observed. The MMCs enriched by Jiang et al. (2012) fed by fermented paper mill wastewater consisted of 56% *Plasticicumulans*, which started to consume acetate and propionate only after the depletion of butyrate. In chapter 3, *Plasticicumulans* was enriched with 27.6% and 50.6% as the most abundant population, under high and low feast DO conditions, respectively. When feeding both cultures the synthetic mixture of the four VFAs for PHA accumulation, butyrate and valerate were always taken up firstly, then acetate and propionate, regardless of the DO levels applied.

In this study, the major difference between the culture selected on day 112 and day 144 was the shift of the percentage of *Aminobacter* and *Plasticicumulans*, besides the elimination of OTU_21 (whose association to PHA storage is unknown)*:* the MMC on the former day being

composed of more *Aminobacter* (11.1%) than *Plasticicumulans* (7.7%), while on the latter day consisting of more *Plasticicumulans* (18.2%) than *Aminobacter* (5.1%). *Aminobacter* was only reported for PHB production by acetate (Kondo et al. 2009), the adaptability to other VFAs is still unknown. Based on literature findings and the tendency observed in this study, *Paracoccus* and *Plasticicumulans* may have had a preference for taking up butyrate and valerate, while *Aminobacter* contributed more towards acetate consumption. Hence, the uptake of VFAs appeared to be more balanced, where acetate, butyrate and valerate were consumed at similar rates on day 112. However, on day 144, the preference for butyrate and valerate was observed to be stronger probably due to the higher proportion of *Plasticicumulans*, which is consistent with the uptake rates presented in Table 5-1. The lower percentage of *Aminobacter* may have led to less acetate consumption when butyrate and valerate were present. It can be hypothesized that higher levels of *Plasticicumulans*, such as those observed in Jiang et al. (2012) and chapter 3, would lead to an even stronger initial butyrate and valerate preference over acetate and propionate, which is consistent with the substrate uptake rates observed in these studies.

5.3.2 The quantification of DO affinity for VFA uptake using the MMCs under high DO conditions.

Since different preferences on the uptake of VFAs were observed in the two MMCs (on day 112 and 144), the DO affinity for the uptake of each VFA may be different as well. The DO affinity for the uptake of each VFA was quantified in Batch_DO_1 and Batch_DO_2, using the cultures selected in both stage 2 (around day 112) and stage 3 (around day 144), with 7 different DO concentrations in the range of 0.1-5 mg/L from assay 1 to assay 7, respectively. The DO concentration of each batch test is listed in Table 5-3.

Table 5-3 DO conditions of the batch tests for the quantification of DO affinity to VFA uptakes for the two MMCs (* the average DO measured in each batch test)

	DO conditions of Batch_DO_1						
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7
DO*	0.10	0.27	0.46	0.74	1.37	2.80	5.02
(mg/L)	±0.05	±0.03	±0.03	±0.08	±0.04	±0.24	±0.05
	DO conditions of Batch_DO_2						
	Assay 1	Assay 2	Assay 3	Assay 4	Assay 5	Assay 6	Assay 7
DO*	0.10	0.27	0.46	0.91	2.28	3.64	5.32
(mg/L)	±0.03	±0.03	±0.05	±0.04	±0.04	±0.03	±0.05

The specific VFA uptake rates at different DO levels from both experimental measurements were fitted by the modified Michaelis-Menten kinetics. Both experimental and simulated results are shown in Figure 5-4. In the Batch_DO_1 (Figure 5-4a), when the DO concentration was lower than 0.5 mg/L, no clear difference of the substrate uptake rates among the four VFAs was

observed. Under unlimited DO conditions, acetate, butyrate and valerate were taken up at the maximum rates around 0.2 Cmol VFA/Cmol X/h, which were slightly higher than the propionate uptake rate 0.13 Cmol VFA/Cmol X/h (Table 5-4). The DO affinity of each VFA uptake was similar according to the K_{DO} values obtained with no statistically difference (Table 5-4).

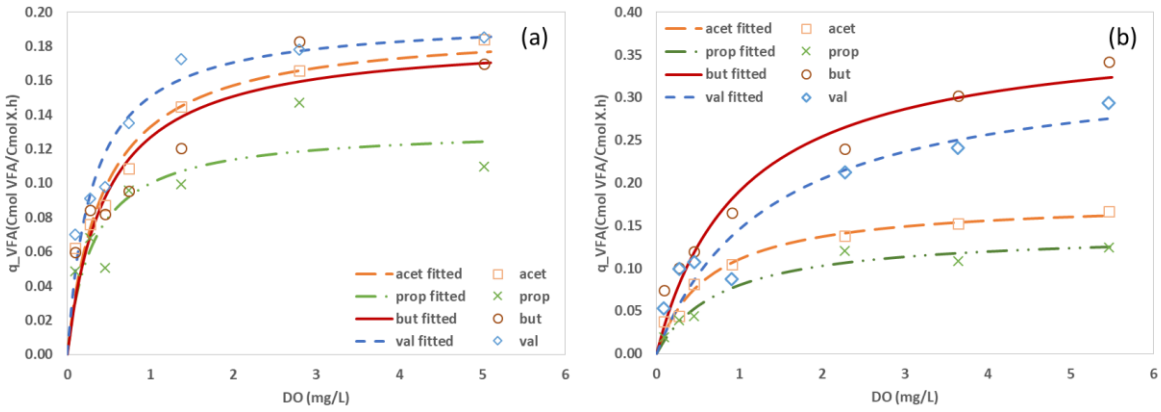

Figure 5-4 Specific VFA uptake rates (q_{VFA}) at different DO levels: (a) Batch_DO_1 and (b) Batch_DO_2. The dots are the rates determined experimentally, and the lines are the fitting curves to the modified Michaelis-Menten kinetics

Table 5-4 q_{max_VFA} and K_{DO} estimated by the modified Michaelis-Menten kinetics

	Batch_DO_1 around day 112		Batch_DO_2 using around day 144	
	q_{max_VFA} (Cmol VFA/Cmol X/h)	K_{DO} (mg/L)	q_{max_VFA} (Cmol VFA/Cmol X/h)	K_{DO} (mg/L)
acetate	0.19±0.01	0.45±0.12	0.18±0.01	0.63±0.11
propionate	0.13±0.02	0.33±0.17	0.14±0.01	0.78±0.23
butyrate	0.19±0.02	0.47±0.19	0.38±0.04	1.01±0.29
valerate	0.20±0.01	0.31±0.08	0.32±0.03	0.92±0.35
total VFAs	0.68±0.05	0.39±0.11	0.92±0.09	0.70±0.27

In the Batch_DO_2 (Figure 5-4b), the uptake rates of the four VFAs were different at each DO level applied. The maximum uptake rates of butyrate and valerate were twice as high as acetate and propionate (Table 5-4). The DO affinities of butyrate and valerate uptake were similar to those of acetate and propionate even though substrate competition occurred, since no significant

statistical difference could be observed for K_{DO} obtained from the simulation (Table 5-4). In spite of the similar K_{DO} results, butyrate and valerate were still the preferred substrate with higher uptake rates regardless of the DO concentration.

In general, the culture selected around day 144 applied to Batch_DO_2 had higher maximum total VFA uptake rate (mainly contributed by butyrate and valerate uptakes), and slightly lower affinity for DO (with slightly higher K_{DO} values for total VFA uptake), as compared to the culture selected around day 112 used for Batch_DO_1 (Table 5-4). It was hypothesized in chapter 3 that butyrate and valerate would be strongly preferred as compared to acetate and propionate, especially under low DO conditions, since the longer chain VFAs required less energy to produce the same amount of PHAs. This preference was clearly observed in Batch_DO_2 by the higher consumption rates of butyrate and valerate. However, in Batch_DO_1, the longer chain VFAs were not always taken up faster than the shorter chain VFAs, for example, the maximum uptake rates of butyrate and valerate were similar to acetate. At low DO levels (less than 10% DO saturation), the four VFAs were taken up at similar rates in Batch_DO_1. Hence, the preference of VFAs is more dependent on the composition of the microbial population, as stated in section 5.3.1, rather than on the energy requirement of individual VFA. The faster consumption of butyrate and valerate than acetate and propionate in Batch_DO_2 could be caused by the increased proportion of *Plasticicumulans* in the selected microbial population, for example.

Based on the results from this study, it seems that the DO concentration is a crucial parameter for successful MMC selection. It was more difficult to enrich the PHA accumulating organisms at low DO levels (0.48±0.29 mg/L) in both feast and famine phases, as compared to the enrichment with low DO concentrations (0.86 ± 0.50 mg/L) only in the feast phase (in chapter 3). Comparing the two studies, in the feast phase, the DO level was lower in the current work, even lower than the K_{DO} (0.70±0.27 mg/L) of the well selected culture in the parallel SBR operated under high DO (3.79±0.65 mg/L) conditions. While in the previous study (chapter 3), due to the larger variation of DO levels, the low DO concentrations applied might be adequate for PHA production and culture enrichment. Hence, it is feasible to select PHA accumulation MMCs under low feast DO conditions (e.g. average DO < 1 mg/L), but the DO concentrations should be not too low to limit the VFA uptake and PHA production activities. In the famine phase, the DO level (3.79±0.65 mg/L) applied in the other study (chapter 3) was much higher than in the current work (DO 0.48±0.29 mg/L). It was reported that low DO levels (close to 0 with no air input) in the famine phase did not impact the PHA accumulation in aerobic granules (Vjayan and Vadivelu 2017). Indeed, since the VFA consumption and PHA production processes are occurred in the feast phase, and the famine phase is mainly responsible for PHA consumption or cell growth and maintenance, which requires far less energy than the feast phase, the impact of DO levels in the famine phase should be less important as compared to the feast phase. Further studies about the influence of the DO concentrations in famine phase on the PHA accumulating culture selection from activated sludge is needed to validate this assumption.

As suggested by this work, the substrate preference is strongly related to the microbial community selected. In the studies with the observation of longer chain VFAs being favored than shorter chain VFAs (Jiang et al. 2012; Marang et al. 2013 and chapter 3), the selected PHA accumulating cultures were all dominated by *Plasticicumulans.* Such strong substrate competition was not reported by any other culture enriched with high population proportion of other PHA accumulating bacteria. Thus, the metabolic model developed in chapter 4 describing the substrate competition is dependent on the composition of the microbial community, and only valid for certain microbial consortia with the same tendency of substrate preference.

5.4 Conclusion

Low DO condition (0.48±0.29 mg/L) in both feast and famine phases was not feasible for long term stable PHA accumulating culture selection, when the DO concentrations limited the VFA uptake and PHA production activities. The substrate preference depended strongly on the composition of the MMCs selected. Butyrate and valerate became the preferred substrates when the percentage of *Plasticicumulans* increased in the selected culture, with higher consumption rates at all DO levels and similar K_{DO} as compared to acetate and propionate. This study suggested that in the MMC enrichment phase, the DO levels should not be limiting. Furthermore, the metabolic model developed in chapter 4 was valid only for MMCs with microbial communities showing the same substrate competition tendency.

References

Albuquerque, Maria G E, Gilda Carvalho, Caroline Kragelund, Ana F Silva, Maria T Barreto Crespo, Maria A M Reis, and Per H Nielsen. 2013. "Link between Microbial Composition and Carbon Substrate-Uptake Preferences in a PHA-Storing Community." *The ISME Journal* (2013) 7,1 (1751–7362/13).

Anterrieu, Simon, Luca Quadri, Bert Geurkink, Inez Dinkla, Simon Bengtsson, Monica Arcos-hernandez, Tomas Alexandersson, et al. 2014. "Integration of Biopolymer Production with Process Water Treatment at a Sugar Factory." *New BIOTECHNOLOGY* 31 (4). Elsevier B.V.: 308–23. doi:10.1016/j.nbt.2013.11.008.

APHA. 1999. "Standard Methods for the Examination of Water and Wastewater." *American Public Health Association*. http://www.mwa.co.th/download/file_upload/SMWW_1000-3000.pdf.

Bengtsson, Simon, Anton Karlsson, Tomas Alexandersson, Luca Quadri, Markus Hjort, Peter Johansson, Fernando Morgan-Sagastume, et al. 2017. "A Process for Polyhydroxyalkanoate (PHA) Production from Municipal Wastewater Treatment with Biological Carbon and Nitrogen Removal Demonstrated at Pilot-Scale." *New Biotechnology* 35 (November). Elsevier B.V.: 42–53. doi:10.1016/j.nbt.2016.11.005.

Carvalho, Gilda, Adrian Oehmen, Maria G E Albuquerque, and Maria a M Reis. 2014. "The Relationship between Mixed Microbial Culture Composition and PHA Production Performance from Fermented Molasses." *New Biotechnology* 31 (4). Elsevier B.V.: 257–63. doi:10.1016/j.nbt.2013.08.010.

Coats, Erik R, Benjamin S Watson, and Cynthia K Brinkman. 2016. "Polyhydroxyalkanoate Synthesis by Mixed Microbial Consortia Cultured on Fermented Dairy Manure : Effect of Aeration on Process Rates / Yields and the Associated Microbial Ecology." *Water Research* 106. Elsevier Ltd: 26–40. doi:10.1016/j.watres.2016.09.039.

Dionisi, D, V Renzi, M Majone, M Beccari, and R Ramadori. 2004. "Storage of Substrate Mixtures by Activated Sludges under Dynamic Conditions in Anoxic or Aerobic Environments" 38: 2196–2206. doi:10.1016/j.watres.2004.01.018.

Dionisi, Davide, Mario Beccari, Simona D. Gregorio, Mauro Majone, Marco Petrangeli Papini, and Giovanni Vallini. 2005. "Storage of Biodegradable Polymers by an Enriched Microbial Community in a Sequencing Batch Reactor Operated at High Organic Load Rate." *Journal of Chemical Technology and Biotechnology* 80 (December 2004): 1306–18. doi:10.1002/jctb.1331.

Dionisi, Davide, Mauro Majone, Giovanni Vallini, Simona Di Gregorio, and Mario Beccari. 2007. "Effect of the Length of the Cycle on Biodegradable Polymer Production and Microbial Community Selection in a Sequencing Batch Reactor." *Biotechnology Progress* 23 (5): 1064–73. doi:10.1021/bp060370c.

Jiang, Yang, Leonie Marang, Jelmer Tamis, Mark C M van Loosdrecht, Henk Dijkman, and Robbert Kleerebezem. 2012. "Waste to Resource: Converting Paper Mill Wastewater to Bioplastic." *Water Research* 46 (17). Elsevier Ltd: 5517–30. doi:10.1016/j.watres.2012.07.028.

Johnson, Katja, Robbert Kleerebezem, and Mark C M van Loosdrecht. 2009. "Model-Based Data Evaluation of Polyhydroxybutyrate Producing Mixed Microbial Cultures in Aerobic

Sequencing Batch and Fed-Batch Reactors." *Biotechnology and Bioengineering* 104 (1): 50–67. doi:10.1002/bit.22380.

Kondo, Takashi, Satoshi Tsuneda, Yoshitaka Ebie, and Yuhei Inamori. 2009. "Characterization of the Microbial Community in the Anaerobic / Oxic / Anoxic Process Combined with Sludge Ozonation and Phosphorus Adsorption" 7 (3): 155–62.

Levantesi, Caterina, Claudia Beimfohr, Bert Geurkink, Simona Rossetti, Karin Thelen, Janneke Krooneman, Jiri Snaidr, Jaap van der Waarde, and Valter Tandoi. 2004. "Filamentous Alphaproteobacteria Associated with Bulking in Industrial Wastewater Treatment Plants." *Systematic and Applied Microbiology* 27 (6): 716–27. doi:Doi 10.1078/0723202042369974.

Marang, Leonie, Yang Jiang, Mark C M van Loosdrecht, and Robbert Kleerebezem. 2013. "Butyrate as Preferred Substrate for Polyhydroxybutyrate Production." *Bioresource Technology* 142 (August). Elsevier Ltd: 232–39. doi:10.1016/j.biortech.2013.05.031.

Oliveira, Catarina S.S., Carlos E. Silva, Gilda Carvalho, and Maria A. Reis. 2016. "Strategies for Efficiently Selecting PHA Producing Mixed Microbial Cultures Using Complex Feedstocks: Feast and Famine Regime and Uncoupled Carbon and Nitrogen Availabilities." *New Biotechnology*. doi:10.1016/j.nbt.2016.10.008.

Pagliano, Giorgia, Valeria Ventorino, Antonio Panico, and Olimpia Pepe. 2017. "Integrated Systems for Biopolymers and Bioenergy Production from Organic Waste and by-Products: A Review of Microbial Processes." *Biotechnology for Biofuels* 10 (1). BioMed Central: 113. doi:10.1186/s13068-017-0802-4.

Queirós, Diogo, Simona Rossetti, and Luísa S Serafim. 2014. "PHA Production by Mixed Cultures: A Way to Valorize Wastes from Pulp Industry." *Bioresource Technology*, no. 0. doi:http://dx.doi.org/10.1016/j.biortech.2014.01.099.

Tamis, Jelmer, Kätlin Lužkov, Yang Jiang, Mark C.M. Van Loosdrecht, and Robbert Kleerebezem. 2014. "Enrichment of Plasticicumulans Acidivorans at Pilot-Scale for PHA Production on Industrial Wastewater." *Journal of Biotechnology* 192: 161–69. doi:10.1016/j.jbiotec.2014.10.022.

Urakami, Teizi, Hisaya Araki, Hiromi Oyanagi, Ken-Ichiro Suzuki, and Kazuo Komagata. 1992. "Transfer of Pseudomonas Aminovorans (Den Dooren de Jong 1926) to Aminobacter Gen . Nov . as Arninobacter Aminovorans Comb . Nov . and Description of Aminobacter Aganoensis Sp . Nov . and Aminobactev Niigataensis Sp . Nov ." *International Journal of Systematic and Evolutionary Microbiology* 42: 84–92.

Vjayan, T, and V M Vadivelu. 2017. "Effect of Famine-Phase Reduced Aeration on Polyhydroxyalkanoate Accumulation in Aerobic Granules." *Bioresource Technology*. doi:10.1016/j.biortech.2017.09.038.

6

APPLICATION OF DISSOLVED OXYGEN LEVEL (DO) CONTROL FOR PHA ACCUMULATION WITH CONCURRENT NITRIFICATION IN SURPLUS MUNICIPAL ACTIVATED SLUDGE

6 APPLICATION OF DISSOLVED OXYGEN LEVEL (DO) CONTROL FOR PHA ACCUMULATION WITH CONCURRENT NITRIFICATION IN SURPLUS MUNICIPAL ACTIVATED SLUDGE

SUMMARY: Mixed microbial cultures are being more widely understood as a viable means for PHA production, which can produce polymers of commercial quality with high yields. Various PHA co-polymer blends can be produced by surplus activated sludge fed by fermented waste feedstocks. The activated sludge has been reported with high PHA storage potential even though it is the surplus biomass from a full-scale municipal biological nitrogen removal process. However, such biomass has high requirement of oxygen from both heterotrophic and autotrophic bacteria, wherein the latter can add extra complexity to process and energy demands since fermented organic residuals are often with significant ammonia concentrations. Dissolved oxygen is the common controlling factor for both metabolisms. Therefore, the influences of DO levels on both PHA accumulation and nitrification rates were studied. The objective was to define a process strategy based on optimal DO control for PHA accumulation, while mitigating the energy input for nitrification activity. A much higher Michaelis–Menten DO affinity for VFA consumption (K_{DO_VFA} 0.1±0.05 mg/L) was found as compared to nitrification (K_{DO_NH4} 0.89±0.01 mg/L). Consequently, by controlling DO levels to lower levels (e.g. 0.4-0.8 mg/L), the PHA production would not be limited by DO, while nitrogen was removed by simultaneous nitrification and denitrification processes. This study has resulted in a proposed strategy for PHA accumulation by nitrifying activated sludge with ammonia containing fermented organic feedstocks by means of DO level control where: (1) nitrification activity and growth are both mitigated and exploited, (2) polymer accumulation is supported by both aerobic and anoxic metabolic activities, and (3) alkalinity is controlled through the maintenance of simultaneous denitrification.

Key words: Polyhydroxyalkanoates (PHAs), biological nutrient removal, nitrification, dissolved oxygen (DO), DO affinity

6.1 Introduction

In the PHA production process, one principal requirement is the supply of PHA accumulating biomass. Mixed microbial cultures (MMCs) were proposed as a cost-effective method to produce biopolymers from renewable resources, wherein through engineered bioprocess conditions, the phenotype of PHA storing microorganisms were naturally enriched (Reis et al. 2011). The surplus activated sludge coming as a by-product from municipal wastewater treatment processes can be a viable source of PHA storing biomass (Bengtsson, et al. 2017b). Enrichment for the PHA storing bacteria in activated sludge biomass is accomplished by subjecting the biomass to periodic alternating environments of feast and famine (van Loosdrecht et al. 1997). Feast conditions can be aerobic or anoxic and entail an environment without organic carbon limitation, whereas famine is generated by the organic carbon limitation.

Aiming at developing commercial PHA production within municipal wastewater treatment, several approaches have been explored. Enrichment of activated sludge with PHA accumulation potential is feasible based on aerobic treatment of organic carbon in wastewater without any acidogenic fermentation step. For example, the municipal wastewater containing readily biodegradable COD (RBCOD) was used directly by Morgan-sagastume et al. (2015) for successful aerobic feast-famine selection of a biomass with significant PHA accumulation potential (34% g-PHA/g-VSS). Nitrogen removal can be combined with biomass selection by pre-denitrification and nitrification processes with the feast phase under anoxic conditions, when treating the municipal and agro-industry wastewaters containing high nitrogen concentrations. Using the surplus activated sludge from biological nutrient removal (BNR) processes for treating municipal wastewater at full-scale for PHA production, as observed by Bengtsson, et al. (2017b), a considerable amount of PHA content around 40-50% (cdw%) was accumulated. Since PHAs were regarded as the by-product with added cascading value from water quality management through wastewater treatment, the integration of the two processes was studied and proved to be favorable also from a life cycle assessment perspective (Morgan-Sagastume et al. 2016). Anterrieu et al. (2014) similarly achieved successful PHA production (>60% g-PHA/g-VSS), integrating PHA production with process water biological nitrogen (and phosphorus) removal at a sugar factory. Bengtsson, et al. (2017a) accomplished high PHA productivity (up to 49% g-PHA/g-VSS) with biological carbon and nitrogen removal from domestic wastewater with an integrated fixed-film activated sludge (IFAS) process at pilot scale.

Enriched biomass harvested from municipal BNR wastewater treatment processes will comprise the populations of PHA storing bacteria as well as ammonia and nitrite oxidizing bacteria (AOBs and NOBs). Meanwhile, the fermented organic residuals being considered as feedstocks for mixed culture PHA accumulation process usually contain a significant amount of ammonia (Basset et al. 2016; Frison et al. 2015). Notwithstanding, certain levels (N/COD: 2-15 mg/g and P/COD: 0.5-3 mg/g) of nutrients addition have been shown to be advantageous with respect to

PHA productivity, without active biomass growth overtaking the PHA storage by the feed-on-demand control activity (Valentino et al. 2015). In fundamental studies of PHA accumulation, inhibiters (thiourea) to the nitrifiers are commonly added to the PHA accumulation feedstock in order to eliminate the activities of AOBs and NOBs (Tamis et al. 2014). In practice, when the addition of inhibiters is not feasible, nitrification may contribute significantly to the overall oxygen demand of the PHA accumulation process. The activities of AOBs and NOBs will also influence the mixed liquor buffering capacity due to the consumption of alkalinity.

Oxygen is the key common substrate for the PHA accumulating heterotrophs and nitrifying autotrophs, which relates directly to the bioprocess aeration and, consequently, energy costs. Since nitrification usually requires higher oxygen levels (Henze et al. 2008), we wished to evaluate the potential of dissolved oxygen control for limiting AOB and NOB activities and to establish the optimal conditions of PHA accumulation in the complexity with the presence of ammonia using a nitrifying activated sludge. To our knowledge, this combination of biomass and substrate has not been evaluated before with the objective to use oxygen supply for the control of heterotrophic and autotrophic microbial activities during mixed culture PHA production. Experiments were carried out using the biomass with significant PHA accumulation potential from a full-scale municipal wastewater treatment plant, to determine the affinity to DO levels for both PHA production and nitrification processes individually. A control strategy was then evaluated with respect to exploiting AOB and NOB activity within a PHA accumulation process, based on a series of laboratory scale experiments using the municipal BNR activated sludge.

6.2 Materials and method

The surplus activated sludge from the Waterboard Brabantse Delta Bath municipal wastewater treatment plant (WWTP) in the Netherlands was used for all the experiments. WWTP Bath is a 470 000 PE plant that receives wastewater via relatively long sewer pressure lines. The wastewater contains contributions from the industries in the area, which is pre-treated by screening and primary settling. The bioprocess configuration includes pre-denitrification and nitrification (also referred as the Modified Ludzack-Ettinger process). Phosphorus removal is mainly done by chemical precipitation (0.42 mol-Me/mol-Pin). The operating conditions promoted a high PHA accumulation potential (40-55% g-PHA/g-VSS) in the surplus activated sludge as reported by Bengtsson et al. (2017b). The grab samples of the activated sludge were obtained from the return sludge channel and stored at 4 °C for no longer than 7 days before use. Before each batch test with grab samples from the WWTP, the sludge was acclimated aerobically for 2 hours at 22°C, in order to establish a base line endogenous aerobic microbial activity.

6.2.1 Batch tests 1: DO levels *versus* rates of PHA accumulation and nitrification

6.2.1.1 Batch tests 1-1: PHA production rates determination at different DO levels

Eight batch tests were carried out in 1L (working volume 800 mL) glass jacketed reactors to determine the PHA production rates at different DO levels. Aeration was provided by air pumps

through ceramic diffusers and magnetic stirring. The DO levels were controlled by adjusting the air mass flow and the values were logged every 10s. The average concentration of DO for each test was estimated from the data record as listed Table 6-1.

To the biomass with VSS about 4.4 g/L, 8 mL substrate mixture (equal amounts of acetate, propionate, butyrate and valerate on Cmol basis) was dosed from concentrated stock to reach 20 Cmmol/L in the batch reactor with no extra nutrients supplied. pH was controlled to be less than 8 (real value 7-8) by dosing HCl (0.5M), and the temperature was maintained at 22 ºC by a water bath. Nitrification was inhibited by adding thiourea in the feed stock together with VFAs to reach 10mg/L in the reactor. In the case of no aeration (effectively zero DO concentration), KNO_3 was added to the reactor (at 20 Nmmol/L) in order to compare the aerobic and anoxic PHA accumulation.

6.2.1.2 Batch tests 1-2: nitrification rates determination at different DO levels

Six batch tests were conducted in 1L (working volume 800 mL) glass jacketed reactors to estimate the nitrification rates at different DO levels (Table 6-1). The equipment, operational conditions and controlling methods for DO, pH and temperature were the same as stated above in the batch tests 1-1 for PHA production rate determinations. Instead of adding VFAs, 8 mL concentrated feedstock containing NH_4Cl and KH_2PO_4 was added to reach 50 mg/L N and 25 mg/L P in the reactor to stimulate nitrification activity.

Table 6-1 DO levels for batch tests

	DO conditions of batch tests 1-1							
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6	Batch 7	Batch 8
DO* (mg/L)	0 (+ nitrate)	0.1 ±0.01	0.3 ±0.07	0.4 ±0.04	1.1 ±0.05	1.5 ±0.19	2.8 ±0.14	4.7 ±0.34
	DO conditions of batch tests 1-2							
	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5	Batch 6		
DO*(mg/L)	0.3 ±0.09	1.3 ±0.06	1.8 ±0.14	2.4 ±0.17	3.1 ±0.17	3.9 ±0.14		

*DO (mg/L) was the average of the measured DO concentrations in each batch test

6.2.2 Estimation of DO level affinity (K_{DO})

The DO affinity of either PHA production or nitrification process was modelled by half-velocity constant with respect to DO (K_{DO}) from modified Michaelis–Menten kinetics (Eq. 6.1): with K_{DO_VFA} for VFA consumption, and K_{DO_N} for ammonium uptake. A higher K_{DO} value suggests a lower affinity towards DO.

$$q = q_{\max} * \frac{C_s}{C_s + K_s} * \frac{C_{DO}}{C_{DO} + K_{DO}}$$

(Eq. 6.1)

$$q \approx q_{\max} * \frac{C_{DO}}{C_{DO} + K_{DO}} \qquad C_S \gg K_S$$

(Eq. 6.2)

q: the specific substrate uptake rate (q_{VFA} or q_{NH4})

q_{max}: the maximum specific substrate uptake rate (q_{max_VFA} or q_{max_NH4})

C_s: the concentration of substrate (VFAs or ammonium)

K_s: half velocity constant of the substrate

C_{DO}: the concentration of DO

K_{DO}: half velocity constant of DO

Since the initial substrate concentrations for both PHA accumulation and nitrification batch tests were much higher than the K_S values, equation 6.1 was simplified as equation 6.2. q_{max} and K_{DO} of each process were estimated by non-linear least squares regression analysis in Sigmaplot (version 12.5) , by fitting the measured q values from the respective batch tests 1 and 2 to equation 6.2.

6.2.3 Batch tests 2: simultaneous PHA production and nitrification at different DO levels

According to the K_{DO_VFA} and K_{DO_N} estimated in batch tests 1, a high DO level (6.0±0.42 mg/L), at which neither PHA production nor nitrification was limited, and a low DO level (0.7±0.05 mg/L), with nitrification inhibited while PHA production not impacted, were determined. These levels were selected to investigate the potential for process control by DO concentrations, for PHA accumulation using substrates comprising significant amount of nutrients as well as organic carbon. Two batch reactors (working volume 500 mL) (batch H and batch L) were operated at the selected high and low DO levels, respectively. The initial concentrations of VFAs (40 Cmmol/L, with equal Cmmol contributions of acetate, propionate, butyrate and valerate) and NH_4Cl (50mgN/L) were established by a single pulse input of the stock solutions into the surplus activated sludge in both batch reactors. The rates and yields for PHA production and nitrification were estimated based on the water quality analyses at each sampling time.

The results were compared to the rate of PHA accumulation from a reference batch test (B_{ref}) that was conducted at high DO levels for 5 hours. In the reference accumulation test, only VFAs (equal amount of acetate, propionate, butyrate and valerate on a Cmol basis) were fed pulse wisely, to maintain the VFA concentration at 20 Cmmol/L after adding each pulse.

6.2.4 Analytical methods

The volatile suspended solids (VSS) were measured according to the standard method (APHA 1999). The filtered samples (through 0.45 µm membrane filter) were used to quantify the concentrations of ammonium, nitrite, and nitrate, by HACH DR6000 UV VIS spectrophotometer

with LANGE cuvette (LCK 303, LCK342 and LCK339, respectively) in batch tests 1, and by a Skalar Scan++ system with a segmented flow analyzer in batch tests 2. The four VFAs (acetate, propionate, butyrate and valerate) and PHAs from batch tests 1 were determined by gas chromatography (GC) as described in Bengtsson et al. (2008). For batch tests 2, VFAs and PHAs were quantified by HPLC and GC, respectively, as elaborated in chapter 3. Since the two series of batch tests were independent from each other, the error between different methods of the measurements was considered negligible.

PHA content was represented in terms of the percentage of biomass VSS: %PHA= PHA/VSS*100 (g PHA/g VSS). Active biomass content during the accumulation process was estimated by subtracting PHA from VSS. The estimated average active biomass content during each batch test was used in further calculations based on the assumption of an elemental composition of $CH_{1.4}O_{0.4}N_{0.2}$ with the theoretical COD 32 g/Cmol (Dias et al. 2005). In the nitrification batch tests 1-2, the active biomass content was the average VSS during each experiment. VFA concentrations are reported as the sum of acetate, propionate, butyrate and valerate concentrations in mgCOD/L. PHA concentrations are similarly reported as the sum of HB and HV in mgCOD/L. The specific VFA consumption rate (q_{VFA} in mgCOD/mgCOD/h) and the specific PHA accumulation rate (q_{PHA} in mgCOD/mgCOD/h) were calculated from the slope of VFA removal or PHA accumulation in the corresponding time period, divided by the corresponding biomass concentration, respectively. The specific ammonium, nitrite and nitrate rates (mgN/mgCOD/h) were calculated by the same method following the slope of the trend from measured values over time. The reported average yield of PHA production on VFA consumption ($Y_{PHA/s}$) was determined from the ratio of q_{PHA} and q_{VFA} on a COD basis.

6.3 Results and discussion

6.3.1 DO affinity for PHA accumulation and nitrification processes

The affinity of DO for either PHA accumulation or nitrification process was identified by the estimation of K_{DO_VFA} and K_{DO_N} following the modified Michaelis–Menten kinetics. As shown in Figure 6-1, the measured trends in specific VFA (Figure 6-1a) and ammonium (Figure 6-1b) consumption rates at different DO levels could be well represented by the modeling results. In order to cross-validate the estimation of K_{DO}, the specific rates of PHA and nitrate production were also simulated by the same method (Figure 6-1c and d). The estimated K_{DO} of VFA consumption was 0.1±0.05 mg/L, while the K_{DO} of NH_4 uptake was 0.89±0.01 mg/L. These two values were replicated with the K_{DO} obtained from PHA and nitrate production, respectively (Table 6-2). Even though nitrite was produced at each DO level, the rates (<0.5 mgN/gCOD/h) were much lower than nitrate production. The much higher K_{DO} values of nitrification process in comparison to PHA production, suggested that nitrifiers had lower affinity to DO than PHA accumulating bacteria. For PHA production, less oxygen was required to reach the maximum rates as compared to the nitrification process. The estimated K_{DO_N} in the present work is within the range (0.3-2 mg/L) of K_{DO} in nitrification process as stated by Henze et al.

(2008), as well as other studies (Ciudad et al. 2006; Blackburne, Yuan, and Keller 2008; Gujer 2010). The absolute values of DO affinities may be affected by the floc size due to the limitations in mass transport by diffusion (Beccari et al. 1992; Henze et al. 2008). Both PHA accumulation and nitrogen conversion rates are functions of temperature (Guo et al. 2013; Grazia et al. 2017). All the present work has been carried out at 22°C and optimal conditions, to minimize the impact of temperature on both nitrification and PHA accumulation activities. This outcome provides an opportunity for using DO controlled to lower levels (e.g. 0.4-0.8 mg/L), in order to regulate nitrification rates without influencing the PHA accumulation activity.

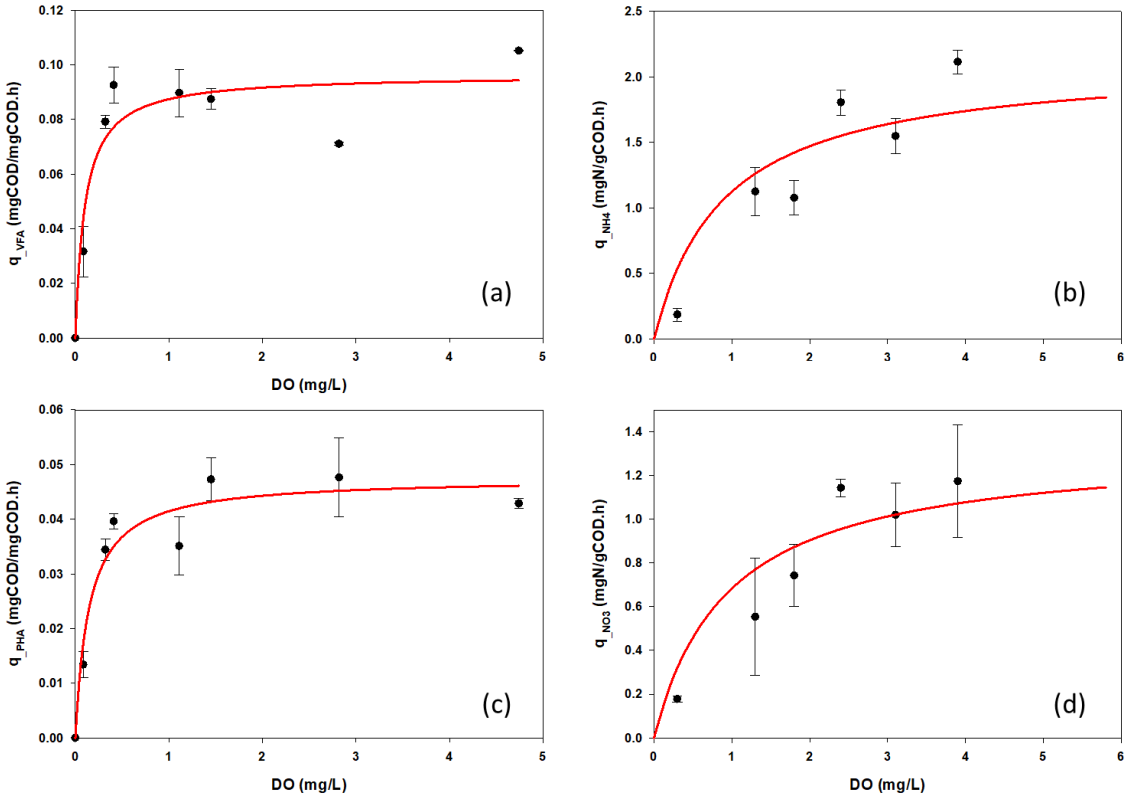

Figure 6-1 (a) Specific VFA uptake rates (q_{VFA}), (b) specific ammonium uptake rate (q_{NH4}), (c) specific PHA accumulation rates (q_{PHA}), and (d) specific nitrate production rate (q_{NO3}) at different DO concentrations. The dots are the rates determined experimentally, and the red lines are the fitting curves to Michaelis–Menten kinetics

Table 6-2 The $K_{\_DO}$ and q estimated in both PHA production and nitrification processes

	PHA production		Nitrification	
	VFA	PHA	NH_4	NO_3
K_{DO} (mg/L)	0.1±0.05	0.15±0.05	0.89±0.01	0.94±0.01
q_{max}*	0.1±0.01	0.05±0.00	2.19±0.09	1.67±0.47

*Units for q_{max_VFA} and q_{max_PHA}: mgCOD/mgCOD/h; for q_{max_NH4} and q_{max_NO3}: mgN/gCOD/h

The yield of PHA production on VFA consumption was not significantly affected by DO change (Table 6-3), where the average yield was 0.49±0.10 mgCOD PHA/mgCOD VFA at all DO levels (except for the anoxic condition). The yield was lower than that from a selected culture fed by the same composition of VFAs (~0.7 mgCOD PHA/mgCOD VFA) as shown in chapter 3, but was similar to that obtained in the PHA accumulation tests using the same surplus activated sludge from the Bath WWTP fed by acetate (0.52 mgCOD PHA/mgCOD VFA) (Bengtsson et al. 2017b).

Under anoxic conditions, PHA was accumulated by denitrifiers using nitrate as the electron acceptor. The specific anoxic VFA consumption rate was 0.06±0.01 mgCOD/mgCOD/h, and the specific anoxic PHA accumulation rate was 0.04±0.01 mgCOD/mgCOD/h. Anoxic VFA uptake rates were estimated to be slightly slower than the aerobic condition, but PHA accumulation rates were nominally the same. The anoxic yield of PHA production on VFA consumption (0.6 mgCOD PHA/mgCOD VFA) was similar to the observed levels obtained aerobically. These values were comparable to another study for PHA accumulation, where the yields were reported as 0.68 mgCOD PHA/mgCOD VFA (0.6 Cmol/Cmol), and 0.45-0.56 mgCOD PHA/mgCOD VFA (0.4–0.5Cmol/Cmol) under aerobic and anoxic condition, respectively (Beun et al. 2002).

Table 6-3 Rates and yields under different DO conditions in PHA production process

DO (mg/L)	0	0.1 ±0.01	0.3 ±0.07	0.4 ±0.04	1.1 ±0.05	1.5 ±0.19	2.8 ±0.14	4.7 ±0.34
q_ VFAs (mgCOD/mgCOD/h)	0.06 ±0.012	0.03 ±0.009	0.08 ±0.002	0.09 ±0.007	0.09 ±0.029	0.09 ±0.004	0.07 ±0.001	0.11 ±0.000
q_ PHA (mgCOD/mgCOD/h)	0.04 ±0.006	0.01 ±0.002	0.03 ±0.002	0.04 ±0.001	0.04 ±0.005	0.05 ±0.004	0.05 ±0.007	0.04 ±0.001
Y PHA/VFAs	0.60	0.42	0.43	0.43	0.39	0.54	0.67	0.41

6.3.2 The demand for DO from both PHA storers and nitrogen converters

6 pulses of VFAs were dosed during a 5h accumulation PHA productivity test (B_{ref}). The VFA consumption rate was 0.12±0.01 mgCOD/mgCOD/h, while the PHA production rate was 0.07±0.01 mgCOD/mgCOD/h. The harvested biomass contained 12.9% (cdw %) PHA, which was a considerable amount for the PHA accumulation using surplus activated sludge, even though the maximum content was not yet reached. This biomass typically reached saturation levels of PHA between 12 and 16 hours of accumulation (25°C) (Bengtsson et al. 2017a). The rates of this B_{ref} were considered to be representative in the range with those obtained in batch tests 1-1, and with the larger pilot study that was undertaken in parallel (Bengtsson et al. 2017a).

Table 6-4 PHA production and nitrification performance rates at low and high DO levels.

PHA production process		Nitrification process		
$q_{\_VFA}$ (mgCOD/mgCOD/h)	$q_{\_PHA}$ (mgCOD/mgCOD/h)	$q_{\_NH4}$ (mgN/gCOD/h)	$q_{\_NO2}$ (mgN/gCOD/h)	$q_{\_NO3}$ (mgN/gCOD/h)

Batch L	-0.11±0.01	0.08±0.00	-2.60±0.41	0.79±0.14	0.08±0.21
Batch H	-0.14±0.02	0.07±0.01	-2.51±0.60	0.44±0.16	2.33±0.29

*Positive values indicate increasing trend and negative values indicate decreasing trend

**PHA content (%) measured at the end of each batch test

The observations of concurrent PHA production and nitrification at high and low DO levels are shown in Figure 6-2. The VFA consumption and PHA production rates were not impacted by the different DO concentrations (Table 6-4), suggesting that both the selected low (0.7±0.05 mg/L) and high (6.0±0.42 mg/L) DO levels were sufficient to not limit PHA production. The low DO level applied in batch L was almost 5 times higher than the K_{DO_VFA} identified in the batch test 1. Rates of PHA production under both high and low DO conditions with ammonium present were similar to the values obtained from the reference batch (B_{ref}) without any nutrients addition. As reported by Frison et al. (2014), nitrification should not impact the PHA yield under aerobic conditions, but alkalinity consumption could increase the variation of pH when dosing acidic fermented substrates.

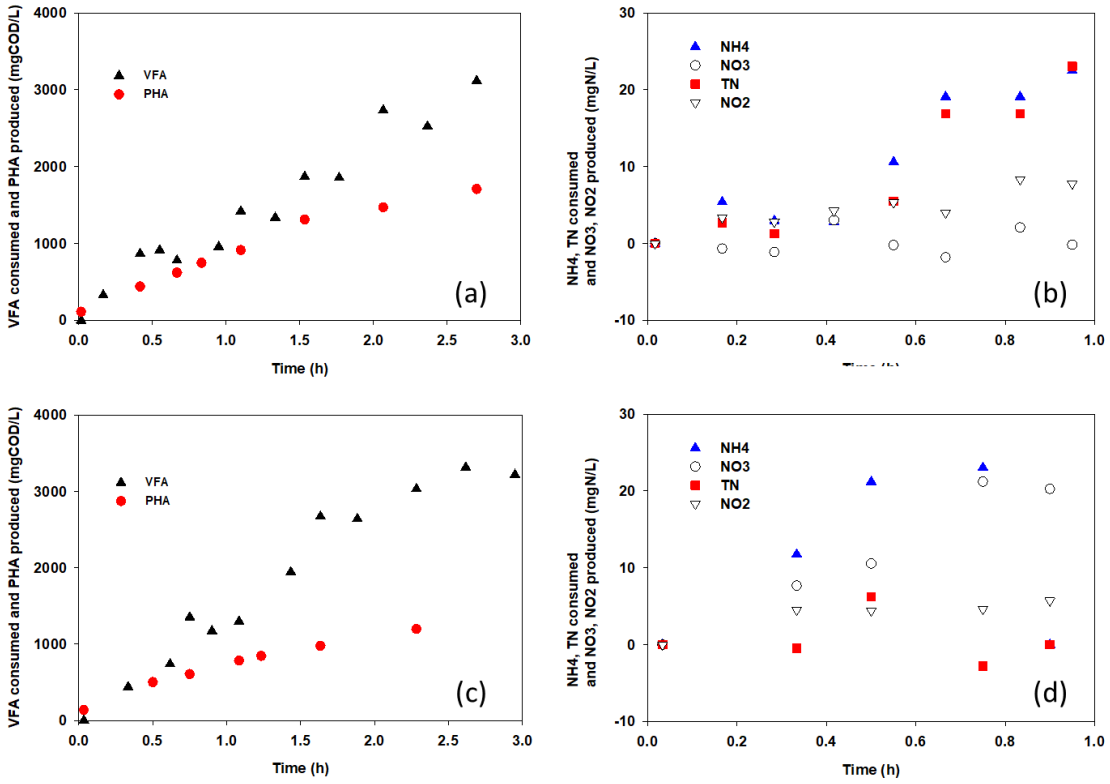

Figure 6-2 At low DO level (Batch L), PHA production performance (a) and nitrification performance (b); at high DO level (Batch H), PHA production performance (c) and nitrification performance (d).

High DO conditions in Batch H resulted in complete nitrification of the added ammonium. The majority of ammonium was converted to nitrate, but some nitrite was accumulated (Figure 6-2d).

With no VFAs addition, similar results were given in batch tests 1-2 at the same DO level. Hence, when DO concentrations were not limiting, both PHA production and ammonium conversion proceeded in parallel. Experience from pilot-scale operation suggested that under these conditions, the observed exponential growth of nitrifying microorganisms ultimately challenged the process dissolved oxygen supply rate (data not shown).

In case of nitrification at low DO levels (Batch L), ammonium was only oxidized to nitrite, instead of being converted further to nitrate (Figure 6-2b). In general, DO concentration should be higher than 2 mg/L for the growth of nitrifiers and the satisfactory nitrification performance (Henze et al. 2008). From previous studies, AOBs were anticipated to exhibit higher oxygen affinity than NOBs (Blackburne et al. 2008; Ciudad et al. 2006). The low DO condition applied in Batch L could therefore be considered to limit the rate of nitrite to nitrate conversion by NOBs, but did not appear to influence the conversion rates to the same extent from ammonium to nitrite by AOBs. In batch tests 1-2, without organic carbon supply, both nitrite and nitrate production were observed at the low DO levels. With diffusion of nitrate and VFAs into the floc, any nitrate produced should in principle be rapidly consumed if the conditions of DO are sufficiently low.

Total nitrogen (TN) was approximately balanced in batch H based on the estimated rates of the nitrification process (Table 6-4). However, in batch L, even though ammonium was consumed at a similar rate as for Batch H, the nitrite accumulation rate was less than half of ammonium consumption rate. The TN concentration decreased at the rate of 2.67±0.67 mgN/gCOD/h. Net nitrite accumulation can be due to oxygen limitation for the NOBs. However, since there was a net loss of total nitrogen, nitrite is anticipated to have been further converted either via denitrification or denitrititation reactions (Pochana and Keller 1999; Frison et al. 2012). Conversion by denitritation would significantly reduce the overall oxygen demand. Since the activated sludge used in this study was enriched from the anoxic-feast and aerobic-famine process in Bath WWTP, heterotrophic denitrifiers that were capable of storing PHA were interpreted to have been selected as demonstrated previously both at laboratory and pilot scale (Anterrieu et al. 2014; Bengtsson et al. 2017a). Simultaneous nitrification and denitrification (SND) via nitrite was similarly observed by Zeng et al. (2003) for low DO levels (0.5 mg/L) during the aerobic phase in the EBPR process. Third et al. (2003) proposed an optimal DO concentration of 1 mg/L towards achieving a maximum SND efficiency and rate, while using acetate for PHB production by a mixed culture. Hence, the low DO levels could regulate the function of NOBs and promote the nitrogen removal by SND, without impacting PHA productivity, as long as the net supply of aerobic or anoxic electron accepters are not limiting.

By controlling the DO at relatively low levels, e.g. 0.4-0.8 mg/L, PHA may be produced at the maximum rates, while nitrogen is converted and removed through SND. The simple strategy of DO level regulation seems to be a viable approach to manage the challenge of PHA accumulation without undue oxygen demands when using nitrogen rich feedstocks. A simultaneous bi-

opolymer production and nitrogen conversion is anticipated to save the energy costs on aeration, while contributing to alkalinity balance in the process. Nevertheless, this study has demonstrated novel principles for PHA production process development. Further work on the principles is required, for example, the influence of the floc size (mass transport) as well as temperature (kinetics and yields), are also important factors that can influence DO optimal level in practice.

6.4 Conclusion

A method to limit the autotrophic activity in a PHA accumulation processes with nutrient rich feedstocks by means of dissolved oxygen control has been developed. It was found that PHA production had a significantly higher affinity to DO in comparison to nitrification, according to estimated Michaelis–Menten K_{DO} values for the respective microbial processes. By applying low DO levels (e.g. 0.4-0.8 mg/L) during PHA accumulation, the PHA production rates were not negatively impacted, while NOB activities were regulated. The advantages of the control strategy include regulating the nitrogen conversion oxygen demand, sustaining balance in the reactor process alkalinity, mitigating excessive growth of NOBs during PHA accumulation process, and managing effluent nitrogen water quality while keeping the aeration energy costs as low as possible.

References

Anterrieu, Simon, Luca Quadri, Bert Geurkink, Inez Dinkla, Simon Bengtsson, Monica Arcos-hernandez, Tomas Alexandersson, et al. 2014. “Integration of Biopolymer Production with Process Water Treatment at a Sugar Factory.” *New BIOTECHNOLOGY* 31 (4). Elsevier B.V.: 308–23. doi:10.1016/j.nbt.2013.11.008.

APHA. 1999. “Standard Methods for the Examination of Water and Wastewater.” *American Public Health Association*. http://www.mwa.co.th/download/file_upload/SMWW_1000-3000.pdf.

Basset, N., E. Katsou, N. Frison, S. Malamis, J. Dosta, and F. Fatone. 2016. “Integrating the Selection of PHA Storing Biomass and Nitrogen Removal via Nitrite in the Main Wastewater Treatment Line.” *Bioresource Technology* 200. Elsevier Ltd: 820–29. doi:10.1016/j.biortech.2015.10.063.

Beccari, M., A. C.Di Pinto, R. Ramadori, and M. C. Tomei. 1992. “Effects of Dissolved Oxygen and Diffusion Resistances on Nitrification Kinetics.” *Water Research* 26 (8): 1099–1104. doi:10.1016/0043-1354(92)90146-U.

Bengtsson, Simon, Anton Karlsson, Tomas Alexandersson, Luca Quadri, Markus Hjort, Peter Johansson, Fernando Morgan-Sagastume, et al. 2017a. “A Process for Polyhydroxyalkanoate (PHA) Production from Municipal Wastewater Treatment with Biological Carbon and Nitrogen Removal Demonstrated at Pilot-Scale.” *New Biotechnology* 35 (November). Elsevier B.V.: 42–53. doi:10.1016/j.nbt.2016.11.005.

Bengtsson, Simon, Alan Werker, Magnus Christensson, and Thomas Welander. 2008. “Production of Polyhydroxyalkanoates by Activated Sludge Treating a Paper Mill

Wastewater." *Bioresource Technology* 99 (3): 509–16. doi:http://dx.doi.org/10.1016/j.biortech.2007.01.020.

Bengtsson, Simon, Alan Werker, Cindy Visser, and Leon Korving. 2017b. "PHARIO Stepping Stone to a Sustainable Value Chain for PHA Bioplastic Using Municipal Activated Sludge."

Beun, J. J., K. Dircks, M. C M Van Loosdrecht, and J. J. Heijnen. 2002. "Poly-B-Hydroxybutyrate Metabolism in Dynamically Fed Mixed Microbial Cultures." *Water Research* 36 (5): 1167–80. doi:10.1016/S0043-1354(01)00317-7.

Blackburne, Richard, Zhiguo Yuan, and Jürg Keller. 2008. "Partial Nitrification to Nitrite Using Low Dissolved Oxygen Concentration as the Main Selection Factor." *Biodegradation*, 303–12. doi:10.1007/s10532-007-9136-4.

Ciudad, G., A. Werner, C. Bornhardt, C. Munoz, and C. Antileo. 2006. "Differential Kinetics of Ammonia- and Nitrite-Oxidizing Bacteria : A Simple Kinetic Study Based on Oxygen Affinity and Proton Release during Nitrification" 41: 1764–72. doi:10.1016/j.procbio.2006.03.032.

Dias, João M.L., Luísa S. Serafim, Paulo C. Lemos, Maria a.M. Reis, and Rui Oliveira. 2005. "Mathematical Modelling of a Mixed Culture Cultivation Process for the Production of Polyhydroxybutyrate." *Biotechnology and Bioengineering* 92 (2): 209–22. doi:10.1002/bit.20598.

Frison, Nicola, Evina Katsou, Simos Malamis, and Francesco Fatone. 2014. "Nutrient Removal via Nitrite from Reject Water and Polyhydroxyalkanoate (PHA) Storage during Nitrifying Conditions," no. July. doi:10.1002/jctb.4487.

Frison, Nicola, Evina Katsou, Simos Malamis, Adrian Oehmen, and Francesco Fatone. 2015. "Development of a Novel Process Integrating the Treatment of Sludge Reject Water and the Production of Polyhydroxyalkanoates (PHAs)." doi:10.1021/acs.est.5b01776.

Frison, Nicola, Silvia Lampis, David Bolzonella, Paolo Pavan, and Francesco Fatone. 2012. "Two-Stage Start-up to Achieve the Stable via-Nitrite Pathway in a Demonstration SBR for Anaerobic Codigestate Treatment." *Industrial and Engineering Chemistry Research* 51 (47): 15423–30. doi:10.1021/ie3009742.

Grazia, Giulia, Luca Quadri, Mauro Majone, Fernando Morgan-Sagastume, and Alan Werker. 2017. "Influence of Temperature on Mixed Microbial Culture Polyhydroxyalkanoate Production While Treating a Starch Industry Wastewater." *Journal of Environmental Chemical Engineering* 5 (5). Elsevier: 5067–75. doi:10.1016/j.jece.2017.09.041.

Gujer, Willi. 2010. "Nitrification and Me - A Subjective Review." *Water Research* 44 (1). Elsevier Ltd: 1–19. doi:10.1016/j.watres.2009.08.038.

Guo, Jingbo, Lanhe Zhang, Wei Chen, Fang Ma, Honglei Liu, and Yu Tian. 2013. "The Regulation and Control Strategies of a Sequencing Batch Reactor for Simultaneous Nitrification and Denitrification at Different Temperatures." *Bioresource Technology* 133. Elsevier Ltd: 59–67. doi:10.1016/j.biortech.2013.01.026.

Henze Mogen, Mark C.M. van Loosdrecht, Ekama George A., and Damir Brdjanovic. 2008. *Biological Wastewater Treatment*.

Morgan-sagastume, F, M Hjort, D Cirne, F Gérardin, S Lacroix, G Gaval, and L Karabegovic. 2015.

"Bioresource Technology Integrated Production of Polyhydroxyalkanoates (PHAs) with Municipal Wastewater and Sludge Treatment at Pilot Scale." *BIORESOURCE TECHNOLOGY* 181. Elsevier Ltd: 78–89. doi:10.1016/j.biortech.2015.01.046.

Morgan-Sagastume, Fernando, Sara Heimersson, Giuseppe Laera, Alan Werker, and Magdalena Svanström. 2016. "Techno-Environmental Assessment of Integrating Polyhydroxyalkanoate (PHA) Production with Services of Municipal Wastewater Treatment." *Journal of Cleaner Production* 137: 1368–81. doi:10.1016/j.jclepro.2016.08.008.

Pochana, Klangduen, and J??rg Keller. 1999. "Study of Factors Affecting Simultaneous Nitrification and Denitrification (SND)." *Water Science and Technology* 39 (6): 61–68. doi:10.1016/S0273-1223(99)00123-7.

Reis, M, M Albuquerque, F C T Universidade, and Nova De Lisboa. 2011. *Comprehensive Biotechnology*. *Comprehensive Biotechnology*. Second Edi. Vol. 1. Elsevier. doi:10.1016/B978-0-08-088504-9.00464-5.

Tamis, Jelmer, Kätlin Lužkov, Yang Jiang, Mark C.M. Van Loosdrecht, and Robbert Kleerebezem. 2014. "Enrichment of Plasticicumulans Acidivorans at Pilot-Scale for PHA Production on Industrial Wastewater." *Journal of Biotechnology* 192: 161–69. doi:10.1016/j.jbiotec.2014.10.022.

Third, Katie A., Natalie Burnett, and Ralf Cord-Ruwisch. 2003. "Simultaneous Nitrification and Denitrification Using Stored Substrate (PHB) as the Electron Donor in an SBR." *Biotechnology and Bioengineering* 83 (6): 706–20. doi:10.1002/bit.10708.

Valentino, Francesco, Lamija Karabegovic, Mauro Majone, Fernando Morgan-sagastume, and Alan Werker. 2015. "Polyhydroxyalkanoate (PHA) Storage within a Mixed-Culture Biomass with Simultaneous Growth as a Function of Accumulation Substrate Nitrogen and Phosphorus Levels." *Water Research* 77. Elsevier Ltd: 49–63. doi:10.1016/j.watres.2015.03.016.

Van Loosdrecht, M. C M, M. a. Pot, and J. J. Heijnen. 1997. "Importance of Bacterial Storage Polymers in Bioprocesses." *Water Science and Technology* 35 (1): 41–47. doi:10.1016/S0273-1223(96)00877-3.

Zeng, Raymond J., Romain Lemaire, Zhiguo Yuan, and Jürg Keller. 2003. "Simultaneous Nitrification, Denitrification, and Phosphorus Removal in a Lab-Scale Sequencing Batch Reactor." *Biotechnology and Bioengineering* 84 (2): 170–78. doi:10.1002/bit.10744.

7

CONCLUSIONS AND FUTURE WORK

7.1 Conclusions

In this thesis, the impacts of DO concentrations on PHA accumulating culture selection stage, PHA accumulation stage, as well as the integration of PHA production with the biological nutrient removal process are generally discussed. The results will contribute to the improvement of process control with low aeration cost by manipulating the DO levels and enhancing the understanding of substrate uptake metabolisms.

MMCs with *Plasticicumulans* as the most abundant population were selected under both high (3.47 ± 1.12 mg/L) and low (0.86 ± 0.50 mg/L) DO conditions in the feast phase. The substrate preference of butyrate and valerate over acetate and propionate was observed in both cultures, but this trend was stronger in the culture selected under low DO conditions, with a higher percentage of *Plasticicumulans* enriched. Even though efficient MMCs were able to be selected at both DO levels, a high DO condition was still essential for the PHA accumulation stage.

A robust metabolic model was developed to describe the substrate competition among the four VFAs (acetate, propionate, butyrate and valerate) in both the feast phase of culture selection and the PHA accumulation stage, based on the observations from the *Plasticicumulans* dominating cultures. This model showed good fitting with experimental data. The stoichiometric and kinetic parameters determined can be used for the prediction of VFA consumption and PHA production where substrate competition occurs.

When operating at a lower DO level (0.48±0.29 mg/L) in both feast and famine phases for the culture selection, the MMCs failed to be selected due to settling problems. Thus, compared to the low DO level (0.86 ± 0.50 mg/L) only in the feast phase, the rather lower DO concentration (0.48±0.29 mg/L) through the entire cycle was less effective for the enrichment of a PHA accumulating culture. The preference of butyrate and valerate over acetate and propionate was observed when the proportion of *Plasticicumulans* increased in a *Paracocccus* dominating (> 50%) culture, indicating that the substrate competition depended strongly on the composition of the microbial communities. It was also observed that this tendency of substrate preference occurred at each DO level from 0.1-5.3 mg/L in the batch tests using this MMC, although the uptake of each VFA showed similar affinity to DO.

DO is the common control factor for both nitrification and PHA production metabolisms. It was found that the nitrifying autotrophs had lower affinity to DO in comparison to the PHA accumulating heterotrophs. Hence, by applying low DO levels (e.g. 0.4-0.8 mg/L) during PHA accumulation, the PHA productivity was not impacted, while the function of NOBs was limited and nitrogen was removed by simultaneous nitrification and denitrification processes. By using this DO control strategy, the energy costs could be saved for PHA production while mitigating the excessive growth of NOBs and managing the effluent water quality in terms of reduced nitrogen content.

7.2 Future work

Based on the work conducted and the results obtained, new questions and suggestions for future work are proposed:

Since the low DO level (<0.5 mg/L) was proved to be insufficient for PHA accumulating culture selection, the lowest threshold value of DO concentration that could promote a successful MMC selection should be identified as a guideline for future studies. The identification of the microbial composition under strictly controlled high and low DO conditions through the entire operational cycle is still worth studying, as the microorganisms selected may be different according to their affinity to DO concentrations. As assumed previously, the impact of DO levels in the famine phase might be less significant compared to that in the feast phase. The validation of this assumption could be conducted by operating two SBRs at the same feast DO levels with different famine DO concentrations. Controlling the DO in the feast and famine at different levels is worth investigating in order to minimize the oxygen input to the system while maintaining an efficient microbial selection.

Due to the complexity of the microbial community structure in MMCs, and the difference between the PHA synthesis mechanisms of different microorganisms, the regulation of the conversion from VFAs to PHAs is not yet fully understood. It is difficult to investigate the individual performance of each population in the MMCs, however, enriched cultures with similar microbial composition should share a common tendency in terms of PHA accumulating activity when subjected to the same conditions. Future metabolic model development could be linked to the microbial profile and specific packages of parameters and stoichiometry should be identified in order to describe accurately the PHA production process, for example, with and without substrate competition. Furthermore, cell growth should be included in the modeling, since PHA synthesis and biomass production occur simultaneously when feeding nutrient rich feedstocks.

The DO control strategy proposed in this study for PHA accumulation with concurrent nitrification in surplus municipal activated sludge provides a new approach for the implementation of simultaneous carbon recovery and nutrients removal. Further questions about the optimization and validation of this strategy still need to be answered, such as the optimal ratio of C/N in the feedstock, the pre-treatment and post-process for complete carbon and nitrogen removal, the mainstream or sidestream PHA accumulation and extraction procedure, the evaluation of the economic values of this strategy, etc. In terms of fundamental research, it would also be interesting to develop a model for the prediction of the PHA production and nitrogen removal efficiency under different DO conditions.

APPENDIX

Appendix A

Chapter 3: microbial community characterization

The most abundant communities (>1%) on day 4 and 32 of culture selection:

One day 4, bacteria with large variety, not selected for PHA accumulation.

Appendix A-1 Most abundant bacteria (>1%) identified by high throughput amplicon sequencing of the cultures enriched in the two SBRs on day 4 of culture selection

Day4	SBR1			SBR2		
	PHYLUM	Specific classification*	%	PHYLUM	Specific classification*	%
1	Actinobacteria	*Gordonia*	8	Actinobacteria	*Gordonia*	7.8
2	Bacteroidetes	QEDR3BF09	7.1	Actinobacteria	*Tetrasphaera*	5.4
3	Chloroflexi	B45	6.6	Chloroflexi	B45	4.6
4	Actinobacteria	*Tetrasphaera*	5.9	Bacteroidetes	QEDR3BF09	4
5	Chloroflexi	C10_SB1A	4.1	Actinobacteria	PeM15	3.2
6	Bacteroidetes	MK04	3.1	Firmicutes	p−55−a5	2.2
7	Firmicutes	p−55−a5	1.8	Chloroflexi	C10_SB1A	1.5
8	Actinobacteria	PeM15	1.6	Bacteroidetes	MK04	1.2
9	Proteobacteria	*Dokdonella*	1.4	Proteobacteria	*Dokdonella*	1.2

* If no genus could be assigned, the best assignment is shown.

On day 32, different microorganisms were selected in low DO SBR compared to high DO SBR, which may be due to the different OLR and SRT in both reactors.

Appendix A-2 Most abundant bacteria (>1%) identified by high throughput amplicon sequencing of the cultures enriched in the two SBRs on day 32 of culture selection

Day 32	High DO SBR			Low DO SBR		
	PHYLUM	Specific classification*	%	PHYLUM	Specific classification*	%
1	Proteobacteria	*Paracoccus*	14.2	Proteobacteria	*Amaricoccus*	26.8
2	Proteobacteria	o__DB1−14_OTU_14	11.7	Proteobacteria	*Azoarcus*	12.3
3	Proteobacteria	*Plasticicumulans*	10.6	Proteobacteria	o__DB1−14_OTU_17	8.7
4	Proteobacteria	f__Hyphomonadaceae_OTU_1	10	Proteobacteria	o__DB1−14_OTU_14	4.9
5	Proteobacteria	*Zoogloea*	8.8	Proteobacteria	*Zoogloea*	13.5
6	Bacteroidetes	*Flavobacterium*	8.4	Bacteroidetes	*Flavobacterium*	12.0
7	Bacteroidetes	f__Cytophagaceae_OTU_50	5	Bacteroidetes	f__Cytophagaceae_OTU_50	4.1
8	Proteobacteria	o__Rhizobiales_OTU_167	4.4	Proteobacteria	*Brevundimonas*	1.9
9	Proteobacteria	*Amaricoccus*	4.1	Proteobacteria	*Aquimonas*	1.8
6	Proteobacteria	Brevundimonas	1.5	Proteobacteria	*Plasticicumulans*	1.4
7	Proteobacteria	*Azoarcus*	1.4			
8	Proteobacteria	o__DB1−14_OTU_17	1.4			

* If no genus could be assigned, the best assignment is shown (f: family; o: order)

Appendix A-3 Most abundant bacteria (>1%) identified by high throughput amplicon sequencing of the cultures enriched in the two SBRs on day 32 of culture selection

	SBR 1			SBR 2		
	Day 4	Day 32	Day 78	Day 4	Day 32	Day 78
Proteobacteria; Plasticicumulans -	0	10.6	27.6	0	1.4	50.6
Proteobacteria; Zoogloea -	0.5	8.8	19.7	0	0.7	13.5
Proteobacteria; Amaricoccus -	0.8	4.1	2	0.5	26.8	0.2
Proteobacteria; Paracoccus -	0.8	14.2	13	0.3	0.6	2.5
Bacteroidetes; Flavobacterium -	0.1	8.4	2.4	0.1	0.3	12
Proteobacteria; o_DB1-14_OTU_14 -	0	11.7	4.8	0.1	4.9	0.5
Proteobacteria; f_Hyphomonadaceae_OTU_1 -	0	10	1.2	0	0.4	5.5
Actinobacteria; Gordonia -	8	0.3	0	7.8	0.1	0
Proteobacteria; Azoarcus -	0	1.4	0.1	0.1	12.3	0.8
Actinobacteria; Tetrasphaera -	5.9	0.3	0	5.4	0.1	0
Chloroflexi; B45 -	6.6	0.4	0	4.6	0	0
Bacteroidetes; f_Saprospiraceae, QEDR3BF09 -	7.1	0.2	0	4	0	0
Proteobacteria; o_DB1-14_OTU_17 -	0	1.4	0	0.1	8.7	0
Bacteroidetes; f_Cytophagaceae_OTU_50 -	0	5	0	0.1	4.1	0.1
Proteobacteria; Meganema -	0.1	0.4	6.7	0.1	0.5	0.3
Chloroflexi; C10_SB1A -	4.1	0.1	0	1.5	0.1	0
Firmicutes; Lactobacillus -	0	0	4.3	1	0	0.1
Proteobacteria; o_Rhizobiales_OTU_167 -	0	4.4	0.1	0	0.4	0
Actinobacteria; PeM15 -	1.6	0.1	0	3.2	0	0
Proteobacteria; Aminobacter -	0	0	3.3	0	0.4	0.7
Proteobacteria; Brevundimonas -	0	1.5	0.3	0.1	1.9	0.7
Bacteroidetes; f_Saprospiraceae, MK04 -	3.1	0.1	0	1.2	0	0
Firmicutes; f_Peptostreptococcaceae, p-55-a5 -	1.8	0	0	2.2	0	0
Protebacteria, Aquimonas -	0	0.8	0.9	0	1.8	0.4
Proteobacteria; Dokdonella -	1.4	0.1	0.3	1.2	0.7	0.1

Appendix A-4 List of the most abundant bacterial groups and the corresponding OTUs in both SBRs on the 3 sampling days (day 4, 32 and 78)

Most abundant bacterial groups	Number of OTUs	OTUs
Proteobacteria; Plasticicumulans -	2	OTU_84; OTU_526
Proteobacteria; Zoogloea -	7	OTU_42; OTU_201; OTU_278; OTU_310; OTU_396; OTU_450; OTU_1245
Proteobacteria; Amaricoccus -	6	OTU_6; OTU_43; OTU_102; OTU_150; OTU_223; OTU_921
Proteobacteria; Paracoccus -	2	OTU_3; OTU_290
Bacteroidetes; Flavobacterium -	20	OTU_20; OTU_76; OTU_188; OTU_197; OTU_258; OTU_426; OTU_527; OTU_557; OTU_614; OTU_671; OTU_738; OTU_822; OTU_871; OTU_872; OTU_886; OTU_1122; OTU_1155; OTU_1356; OTU_1365; OTU_1401
Proteobacteria; o_DB1-14 -	17	OTU_5; OTU_14; OTU_17; OTU_34; OTU_119; OTU_153; OTU_185; OTU_233; OTU_268; OTU_398; OTU_476; OTU_690; OTU_717; OTU_801; OTU_915; OTU_973; OTU_1011
Proteobacteria; f_Hyphomonadaceae -	4	OTU_1; OTU_144; OTU_607; OTU_1053
Actinobacteria; Gordonia -	4	OTU_40; OTU_55; OTU_203; OTU_633
Proteobacteria; Azoarcus -	2	OTU_27; OTU_129
Actinobacteria; Tetrasphaera -	4	OTU_13; OTU_122; OTU_316; OTU_1051
Chloroflexi; B45 -	2	OTU_31; OTU_136
Bacteroidetes; f_Saprospiraceae, QEDR3BF09 -	9	OTU_46; OTU_72; OTU_303; OTU_382; OTU_449; OTU_641_OTU_786; OTU_819; OTU_1290

Bacteroidetes; f_Cytophagaceae -	27	OTU_28; OTU_50; OTU_98; OTU_127; OTU_166; OTU_168; OTU_219; OTU_240; OTU_252; OTU_286; OTU_341; OTU_546; OTU_573; OTU_575; OTU_579; OTU_730; OTU_744; OTU_814; OTU_1014; OTU_1016; OTU_1081; OTU_1164; OTU_1200; OTU_1304; OTU_1334; OTU_1420; OTU_1428
Proteobacteria; Meganema -	4	OTU_61; OTU_202; OTU_214; OTU_1437
Chloroflexi; C10_SB1A -	1	OTU_41
Firmicutes; Lactobacillus -	5	OTU_47; OTU_253; OTU_599; OTU_1180; OTU_1342
Actinobacteria; PeM15 -	4	OTU_113; OTU_165; OTU_976; OTU_1273
Proteobacteria; Aminobacter -	1	OTU_10
Proteobacteria; Brevundimonas -	3	OTU_15; OTU_37; OTU_215
Bacteroidetes; f_Saprospiraceae, MK04 -	2	OTU_26; OTU_79
Firmicutes; f_Peptostreptococcaceae, p-55-a5 -	2	OTU_176; OTU_435
Protebacteria, Aquimonas -	2	OTU_25; OTU_1429
Proteobacteria; Dokdonella -	3	OTU_87; OTU_246; OTU_953

Appendix A-5 Number of reads after DNA sequencing, QC and bioinformatics processing

Samples		Number of Reads
SBR1	Day 4	26061
	Day 32	4515
	Day 78	25084
SBR2	Day 4	19339
	Day 32	37063
	Day 78	20644

The preparation of the samples for DNA sequencing, using the V1-3 16S rRNA bacterial primers was successful. The PCR conditions consisted in 10 ng of extracted DNA used as template and 25 μL of the PCRmix with 400 nM of dNTPs (each), 1.5mM of MgSO4, 2mU of Platinum® Taq DNA polymerase HF, 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA), and 400nM of barcoded library adaptors with the V1-3 primers: 27F AGAGTTT-GATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG. PCR settings: Initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s and final elongation at 72°C for 5 min. The reads yielded between 4515 and 37063 after QC and bioinformatic processing.

Appendix B

Chapter 4: mATP estimation

500ml waste activated sludge from the end of the famine phase of SBR1 (high feast DO) was inoculated in 2 batch reactors, with the same DO concentrations as the accumulation tests B1(HH) and B2 (HL). Similarly, the same amount of wasted sludge from SBR2 (low feast DO) was inoculated in the other 2 batch reactors mimicking the same DO conditions as B3 (LH) and B4 (LL). The only difference of the batch tests for mATP estimation from the accumulating batches was that no external carbon was supplied. No cell growth occurred due to the lack of ammonia availability. PHAs that were stored in the cells during the feast phase were consumed to maintain the metabolism of cells. Hence, mATP was predicted by the consumption rate of PHAs.

The overall reactions and stoichiometry for mATP estimation are shown in Appendix B-1. The intracellular intermediates and energy (acetyl-CoA, propionyl-CoA, ATP and $NADH_2$) balance at steady state are listed in eq.(A1)- (A4):

Acetyl-CoA: $R_1' + 0.4R_2' - R_5' + R_6' = 0$ (A1)

Propionyl-CoA: $0.6R_2' - 1.5R_6' = 0$ (A2)

ATP: $\delta R_3' - R_4' + 0.5R_5' = 0$ (A3)

NADH2: $0.25R_1' + 0.2R_2' - R_3' + 2R_5' + 1.5R_6' = 0$ (A4)

Hence, m_{ATP} can be expressed by PHA consumption rates as eq. (A5).

$$m_{ATP} = R_4' = 5R_1' + 5.2R_2' \quad \text{(A5)}$$

Appendix B-1 Reactions and stoichiometry for mATP estimation through post-famine experiments

	Reaction	stoichiometry
R_1'	PHB degradation	$CH_{1.5}O_{0.5} \rightarrow CHO_{0.5} + 0.25NADH_2$
R_2'	PHV degradation	$CH_{1.6}O_{0.4} \rightarrow 0.4CHO_{0.5} + 0.6CH_{4/3}O_{1/3} + 0.2NADH_2$
R_3'	Oxidative Phosphorylation	$NADH_2 + 0.5O_2 \rightarrow H_2O + \delta ATP$
R_4'	Maintenance	$ATP \rightarrow m_{ATP}$
R_5'	Catabolism	$CHO_{0.5} + 1.5H_2O \rightarrow CO_2 + 2NADH_2 + 0.5ATP$
R_6'	Decarboxylation	$1.5CH_{4/3}O_{1/3} + H_2O \rightarrow CHO_{0.5} + 1.5NADH_2 + 0.5CO_2$