# Polyhydroxyalkanoate production by municipal waste activated sludge

## Tackling scaling-up process challenges

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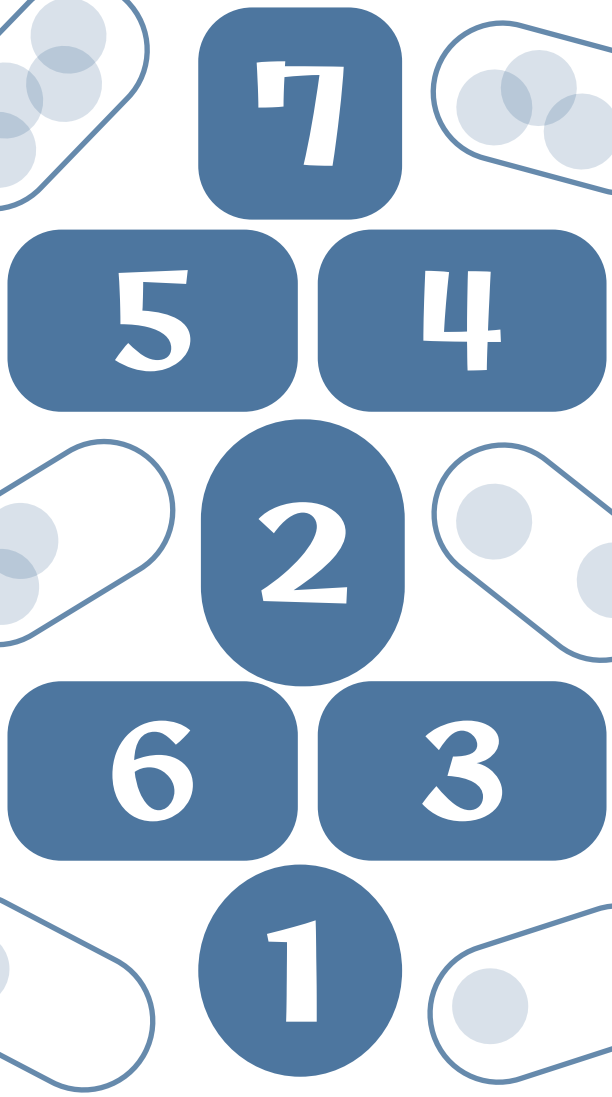


Ángel Estévez Alonso

# Propositions

accompanying the dissertation

**POLYHYDROXYALKANOATE PRODUCTION BY MUNICIPAL WASTE ACTIVATED SLUDGE**
TACKLING SCALING-UP PROCESS CHALLENGES

by

**Ángel ESTÉVEZ ALONSO**

*These propositions pertain to this dissertation*

1. Waste activated sludge should not be considered a waste stream anymore.
2. Biomass growth is beneficial for polyhydroxyalkanoate production in polyhydroxyalkanoate accumulation reactors.
3. The presence of flanking microbial populations is not detrimental for mixed culture polyhydroxyalkanoate production.

*These propositions do not pertain to this dissertation*

4. In a scientific article, writing a combined "Results and Discussion" section is a sign that the author has not a real discussion point.
5. Many scientific discoveries go unnoticed because an observation did not agree with the researcher's previous mindset.
6. Circular economy objectives can only be achieved with multidisciplinary research cooperation.
7. Success is determined by being at the correct place at the right moment.
8. The current obsession with the pursuit of excellence creates huge pressure in young people minds. Not everybody needs to be excellent.
9. We should all learn how to realistically manage our own expectations and deal with failure.

These propositions are regarded as opposable and defendable,
and have been approved as such by the promotors and co-promotor
Prof. dr. ir. M.C.M. van Loosdrecht, dr. ir. R. Kleerebezem and dr. A.G. Werker.

# POLYHYDROXYALKANOATE PRODUCTION BY MUNICIPAL WASTE ACTIVATED SLUDGE

## TACKLING SCALING-UP PROCESS CHALLENGES

# POLYHYDROXYALKANOATE PRODUCTION BY MUNICIPAL WASTE ACTIVATED SLUDGE

TACKLING SCALING-UP PROCESS CHALLENGES

## Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
by the authority of the Rector Magnificus Prof. dr. ir. T.H.J.J. van de Hagen,
chair of the Board for Doctorates,
to be defended publicly on
Thursday 1st December 2022 at 15:00 h.

by

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This dissertation has been approved by the

promotor: Prof. dr. ir. M.C.M. van Loosdrecht
promotor: Dr. ir. R. Kleerebezem
copromotor: Dr. A.G. Werker

Composition of the doctoral committee:

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|---|---|
| Rector Magnificus | chairperson |
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| Dr. ir. R. Kleerebezem | Technische Universiteit Delft |
| Dr. A.G. Werker | Wetsus |
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| Prof. dr. A. Mosquera Corral | University of Santiago de Compostela |
| Prof. dr. ir. M. K. de Kreuk | Technische Universiteit Delft, *reserve* |

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# Contents

# Summary

Microbial life is all around us. Microbes are the gears that sustain all forms of life in planet Earth and are expected to become key players in the transition towards a more sustainable society. In this thesis, microbes from sewage sludge are used for the production of polyhydroxyalkanoates, biodegradable polymers that have the potential to replace many of the petroleum-based plastics that are used nowadays. A general overview of the role of microorganisms in nature cycles and how they can be used for the production of useful chemicals in a bio-based circular economy is presented in **Chapter 1**.

In **Chapter 2**, the focus is given to the current status of microbial community-based PHA production. Microbial community-based PHA production has shown an increase in pilot-scale installations over the past 10 years and it seems ready for further scaling-up. Scaling-up challenges are mainly related to the polymer recovery methods and the development of new applications for the extracted polymer. However, alternative biological PHA production routes are still under development. For instance, the direct use of municipal waste activated sludge for PHA production. Consequently, this thesis aims to address the current scaling-up challenges of PHA production by municipal waste activated sludge.

The developments of mixed culture PHA production has been directed to optimal operational conditions to maximize the biomass PHA content with limited attention to polymer quality. Moreover, direct comparison of PHA accumulation literature is challenging, and even regularly contradicting in reported results. In **Chapter 3**, a comparison is made between the most commonly reported operational conditions in PHA production with municipal activated sludge. A biomass acclimation step combined with a pulse-wise feeding strategy resulted in maximum average PHA contents and product yields while pH control and the presence of nitrifying activity did not result in observable effects on the PHA productivity. Under these conditions a high molecular weight polymer (1536 ± 221 kDa) can be

produced. A protocol has been made available for standardised PHA accumulation tests with municipal activated sludge.

**Chapter 4** explores the limits of PHA production by municipal activated sludge. Standardised PHA accumulation tests with microscopic evaluation were performed with municipal activated sludge from six different WWTPs. The degree of enrichment for PHA-accumulating bacteria was identified as the key factor to maximise PHA contents when municipal activated sludge is directly used for PHA production. Future optimisation should focus on obtaining a higher degree of enrichment of PHA accumulating biomass, either through selection during wastewater treatment or by selective growth during PHA production. A PHA content in the order of 0.6 gPHA/gVSS is a realistic target to be achieved when using municipal activated sludge for PHA production.

In **Chapter 5**, a strategy that couples the selective growth of PHA-storing bacteria with PHA accumulation to maximized PHA production is proposed. It was found that the presence of calcium promotes selective growth and enrichment of the PHA-storing biomass fraction and significantly improve both PHA contents and yields. After 48 h, three times more PHA was produced compared to control experiments without calcium addition. Higher PHA content and selective biomass production is proposed to be a consequence of calcium dependent increased levels of passive acetate uptake. These findings lead to bioprocess methods that enable improvements to the techno-economic feasibility of municipal waste activated sludge to become a generic resource for industrial scale PHA production.

**Chapter 6** is focused on the mitigation of flanking microbial populations that are not actively involved in the production of PHA. In this chapter, the use of low dissolved oxygen concentrations was evaluated as a means to minimize the activity of nitrifying bacteria. It was found that nitrifying bacteria were more affected than PHA-storing bacteria by low dissolved oxygen concentrations. Based on these results, it was proposed the use of low dissolved oxygen concentrations to limit the activity of flanking microbial populations in PHA production reactors.

**Chapter 7** describes the main conclusions of this thesis and discuss the future research and remaining scaling-up challenges of microbial community-based PHA production.

# Samenvatting

Microbieel leven is overal om ons heen. Micro-organismen zijn de tandwielen die alle vormen van leven op planeet aarde in stand houden en zullen naar verwachting belangrijke spelers worden in de transitie naar een duurzamere samenleving. In dit proefschrift worden micro-organismen uit surplus actief slib gebruikt voor de productie van polyhydroxyalkanoaten. Dit zijn biologisch afbreekbare polymeren die de potentie hebben om op aardolie gebaseerde kunststoffen te vervangen. Een algemeen overzicht van de rol van micro-organismen in natuurkringlopen en hoe ze kunnen worden gebruikt voor de productie van nuttige chemicaliën in een circulaire biobased economie wordt gegeven in **Hoofdstuk 1**.

In **Hoofdstuk 2** wordt de focus gelegd op de huidige status van microbiële gemeenschap-gebaseerde PHA productie. Het aantal installaties met microbiële gemeenschap-gebaseerde PHA productie op proefschaal is de afgelopen 10 jaar toegenomen en de techniek lijkt klaar voor verdere schaalvergroting. Opschalingsuitdagingen hebben voornamelijk betrekking op de polymeerterugwinningsmethoden en de ontwikkeling van nieuwe toepassingen voor het geëxtraheerde polymeer. Er zijn echter nog ontwikkelingen gaande op het gebied van alternatieve biologische PHA productieroutes. Bijvoorbeeld het gebruik van surplus actief slib uit gemeentelijke rioolwaterzuiveringsinstallaties (RWZI's) voor de productie van PHA. Dit proefschrift richt zich op de huidige opschalingsuitdagingen van PHA productie met surplus actief slib uit RWZI's.

De ontwikkelingen van PHA productie met gemengde culturen zijn gericht op optimale operationele omstandigheden om het PHA gehalte van de biomassa te maximaliseren, met beperkte aandacht voor polymeerkwaliteit. Bovendien is directe vergelijking van literatuur over PHA accumulatie een uitdaging, en zelfs regelmatig in tegenspraak met elkaar. In **Hoofdstuk 3** wordt een vergelijking gemaakt tussen de meest gerapporteerde operationele omstandigheden in PHA productie met surplus actief slib uit RWZI's. Een biomassa-acclimatiseringsstap ge-

combineerd met een pulsgewijze voedingsstrategie resulteerde in maximale gemiddelde PHA gehalten en productopbrengsten, terwijl pH regeling en de aanwezigheid van nitrificerende activiteit niet resulteerden in waarneembare effecten op de PHA productiviteit. Onder deze omstandigheden kan een polymeer met een hoog molecuulgewicht (1536 ± 221 kDa) worden geproduceerd. Er is een protocol beschikbaar gesteld voor gestandaardiseerde PHA accumulatietesten met surplus actief slib uit RWZI's.

**Hoofdstuk 4** verkent de grenzen van de PHA productie door surplus actief slib uit RWZI's. Gestandaardiseerde PHA accumulatietesten zijn uitgevoerd met surplus actief slib van zes verschillende RWZI's. De mate van verrijking voor PHA accumulerende bacteriën in de microbiële gemeenschap werd geïdentificeerd als belangrijkste factor om PHA gehalten te maximaliseren. Toekomstige optimalisatie moet gericht zijn op het verkrijgen van een grotere fractie PHA accumulerende bacteriën in de biomassa, hetzij door selectie tijdens het afvalwaterzuiveringsproces op de RWZI, hetzij door selectieve groei tijdens PHA productie. Een PHA gehalte in de orde van grootte van 0.6 gPHA/gVSS is een realistisch streefdoel bij PHA productie met surplus actief slib uit RWZI's.

In **Hoofdstuk 5** wordt een strategie voorgesteld voor de selectieve groei van PHA accumulerende bacteriën tijdens het PHA productieproces, om zo de productie van PHA te maximaliseren. De aanwezigheid van calcium blijkt de selectieve groei van de PHA producerende biomassa-fractie te bevorderen waardoor zowel het PHA gehalte als de opbrengst aanzienlijk wordt verhoogd. Na 48 uur werd drie keer meer PHA geproduceerd in vergelijking met controle-experimenten zonder toevoeging van calcium. Er wordt aangenomen dat een hoger PHA gehalte en selectieve biomassaproductie een gevolg zijn van calcium-afhankelijke verhoogde niveaus van passieve acetaatopname. Deze bevindingen leiden tot bioproces-methoden die de technisch-economische haalbaarheid kunnen vergroten en waarbij surplus actief slib uit RWZI's een algemene grondstof kan worden voor PHA productie op industriële schaal.

**Hoofdstuk 6** is gericht op het verminderen van flankerende microbiële populaties die niet actief betrokken zijn bij de productie van PHA. In dit hoofdstuk werd het gebruik van lage concentraties opgeloste zuurstof geëvalueerd als middel om

de activiteit van nitrificerende bacteriën te minimaliseren. Het bleek dat nitrificerende bacteriën meer werden geremd dan PHA accumulerende bacteriën door lage concentraties opgeloste zuurstof. Op basis van deze resultaten word voorgesteld om lage concentraties opgeloste zuurstof te gebruiken om de activiteit van flankerende microbiële populaties in PHA productiereactoren te beperken.

**Hoofdstuk 7** beschrijft de belangrijkste conclusies van dit proefschrift en bespreekt het toekomstig onderzoek en de resterende opschalingsuitdagingen van microbiële gemeenschap-gebaseerde PHA productie.

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# Preface

In the famous book Rayuela (Hopscotch in English), the Argentinian writer Julio Cortázar presents the reader with two different ways of reading his book. The reader can choose to follow either a linear and ordinary way or an alternative and more chaotic approach. Here, I would also like to invite the reader to play with this thesis, as Cortázar did in his book. The reader can choose to read this thesis in the following two ways.

1. This thesis can be read in an ordinary way, from Chapter 1 to Chapter 7.

2. Alternatively, this thesis can also be read in the same order the writer performed the experiments. The reader can start in Chapter 1, followed by Chapter 6, Chapter 3, Chapter 2, Chapter 5, Chapter 4 and Chapter 7. For simplicity, at the end of each chapter there is an indication for the following chapter.

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# 1

# Introduction

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## 1.1. Circular economy objectives and nature-based solutions

By 2050, the global consumption of biomass, fossil fuels, metals and minerals will double and consequently, an increase of 70% in annual waste generation is expected (Kaza et al., 2018; OECD, 2019; Commission, 2020). This increase is expected to result also in significant greenhouse gas emissions and biodiversity losses. The European Union looks to tackle these challenges. In March 2020 the European Union adopted the new circular economy action plan. This plan aims to achieve the European Union climate neutrality and to halt biodiversity losses (Commission, 2020). In the new circular economy action plan, special attention is given to the reduction of waste, to the recovery of useful resources from waste, and a transition towards a bio-based society. Nature-based solutions are expected to enable European countries to reach the sustainable development goals proposed in the new circular economy action plan (Davies et al., 2021; Ghafourian et al., 2021; Langergraber et al., 2020).

Nature is a perfect example of a bio-based society with zero waste generation. By-products of one metabolism are usually used by a subsequent metabolism from another organism (Madigan et al., 2010). For instance, $CO_2$ is captured from the atmosphere by plants and/or photosynthetic microorganisms for the production of molecules with longer carbon chains. These complex molecules are subsequently used by the same or other organisms for their growth. Usually, the cycle is closed when these complex molecules are fully oxidized for energy generation with concurrent $CO_2$ production. In such examples, material flows are in perfect balance and everything that is produced by one organism or process cascades to be used by the subsequent.

## 1.2. Microorganisms are all around us

Microbial life is all around us. Among other places, microorganisms are present in the food we eat, in the clothes we wear, in the soil we tramp on and in the water we drink (Probandt et al., 2018; Lewis et al., 2021). Microorganisms can be present theoretically anywhere where energy can be harvested as part of a chemical reaction and liquid water exists. Among other things, microorganisms are respon-

sible for up to 20% of $O_2$ present in our biosphere, constitute from 0.3 to 3% of human weight, and drive of all Earth's biogeochemical cycles (Sender et al., 2016; Falkowski et al., 2008). Consequently, microbial life is intertwined with human life. Historically, microorganisms have had a bad reputation, because they are also responsible for many forms of disease (Madigan et al., 2010). However, in the late $19^{th}$ century, microorganisms were also found to be responsible for many kinds of harmless transformation processes and since then, broader perceptions, with wonder and respect, for microbial life have grown.

Microorganisms have been used both intentionally and unintentionally by humankind for many purposes. Already at the end of the Pleistocene and start of the Holocene microbial fermentations were used to preserve food (Craig, 2021). For instance, ancient Chinese cultures were among the first producers of fermented beverages (McGovern et al., 2004). Nowadays, microorganisms are routinely used for production of valued chemicals and even for the treatment of different waste streams in environmental protection. Microorganisms and microbial metabolic activities are expected to play a central role on the road towards meeting circular economy objectives (Nielsen, 2017; Schilling and Weiss, 2021; Leipold and Petit-Boix, 2018; Tan and Lamers, 2021).

## 1.3. Microorganisms in a bio-based society

Traditionally, defined strains of microorganisms have been used in bioprocesses for the production of many kinds of chemicals such as ethanol or pharmaceuticals. However, these classical biotechnology processes require refined substrates for feedstocks and sterile starting conditions to avoid contamination by unwanted microbial strains (Tang and Zhao, 2009). The combination of purified substrates and sterilization contributes to make production costs high. Additionally, contamination can be an even bigger challenge to avoid when waste streams are used as cheaper substrates for the production of the same chemicals (Rodriguez Perez et al., 2018).

Unlike industrial biotechnology, environmental biotechnology aims at designing and operate process environments that select for strains with desired conversions rather than designing and culturing specific microorganisms under axenic

conditions. Environmental biotechnological processes have been instrumental for many years in the removal of generic and specific polluting compounds from municipal and industrial waste streams so that effluents enter back in nature without detrimental ecological or environmental effects (Kleerebezem et al., 2015). An already classic example is the activated sludge process for the treatment of municipal wastewater (Lofrano and Brown, 2010). In an activated sludge process, microbial biomass, known as activated sludge, uses the carbon, nitrogen and phosphorus present in the wastewater for growth purposes. In this way, cleaner water is produced. However, as result of microbial growth while removing contamination, a waste by-product in the form of microbial biomass is produced. This waste microbial by-product receives the name of waste activated sludge. The waste activated sludge is nowadays considered a waste stream and its disposal contributes significantly to the wastewater treatment operational costs. Nonetheless, waste activated sludge can instead be a resource for the production of biopolymers (Arcos-Hernandez et al., 2013).

In the past decades, environmental biotechnology has also been harnessed for the efficient production of specific organic products. Towards this end, a combination of industrial and environmental biotechnology methods has been proposed. The combination of these two disciplines has been called 'mixed culture biotechnology' or 'microbial community engineering' (Kleerebezem and van Loosdrecht, 2007). Microbial community engineering relies on the use of ecological selection principles for the enrichment of consortia of microbial communities with specific functionalities to produce platform chemicals as products. Microbial community engineering is founded in the proposition from the Dutch botanist Baas-Becking that 'Everything is everywhere, but the environment selects' (Becking, 1934). Therefore, in microbial community engineering a deep understanding of the ecological role of the product of interest is required to drive the selection towards microbial communities with potential in the formation of such products. Nowadays, microbial community engineering principles have been successfully applied at different scales for the production of short and long-chain fatty acids and biopolymers such as polyhydroxyalkanoates or alginate like polymers (Angenent et al., 2016; Kourmentza et al., 2017; Feng et al., 2021).

## 1.4. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA) are a group of biodegradable polyesters generated by bacteria as energy and carbon storage reserves (Dawes and Senior, 1973). PHAs have attracted research attention due to their biodegradability and potential as a bio-based renewable alternative to petrochemical polymers (Philip et al., 2007; Fernández-Dacosta et al., 2015). PHAs can exhibit thermoplastic properties similar to polypropylene and polyethylene. Thus, PHAs can be used in a way like plastics, but in applications where biodegradability plays an important role. In bacteria, PHAs are stored as intracellular granules when bacteria are exposed to dynamic environmental conditions (Van Loosdrecht et al., 1997). PHA storage metabolism evolved as a microbial survival strategy due to environments with periodic presence and absence of electron acceptor, carbon source and/or growth nutrients. Currently, PHAs are produced using industrial biotechnology methods and defined bacterial strains that can intracellularly accumulate PHAs up to 90% of their cell dry weight (Koller, 2018). In the PHA accumulation reactor, bacteria produce PHA until there is no more available intracellular space and the maximum cellular PHA content is obtained. In pure cultures, these maximum PHA contents are achieved with the use of nitrogen limitation to restrict the growth response of the microorganisms and promote the accumulation of PHA. Using industrial biotechnology methods, it is estimated that 25.3 kt PHA are produced yearly (Bioplastics, 2020; Vandi et al., 2018).

In the past two decades, microbial community engineering principles have also been explored extensively as an effective technology for PHA production. PHA-storing bacteria can be enriched by applying selective process conditions that favour PHA-producing microorganisms over non-PHA accumulating microorganisms (Reis et al., 2003). By applying an alternating presence and absence of the carbon source, microorganisms that are able to accumulate PHAs when carbon is present (feast) increase in relative proportions due to a survival advantage during the longer periods without substrate (famine). The enrichment of PHA-storing bacteria is usually performed in the so-called *selection* reactor. Subsequently, the biomass produced in the *selection* reactor is used in the *accumulation* reactor to maximize the cellular PHA content of the PHA-storing biomass. Mixed culture

PHA production at laboratory and pilot scale can achieve a comparable cellular PHA content, as those that have been obtained with pure culture methods (Sabapathy et al., 2020; Kourmentza et al., 2017). In this approach, part of the feedstock is used not only for PHA production, but also for the enrichment of PHA-storing bacteria. To overcome this challenge, it has been proposed to directly use municipal waste activated sludge for PHA production. Dynamic environments often observed in municipal wastewater treatment plants tend to enrich for microorganisms that are able to store intracellular compounds as carbon or energy reservoirs. A municipal wastewater treatment is not designed for the production of intracellular polymers, but a significant fraction of the municipal activated sludge is composed of PHA-storing microorganisms (Van Loosdrecht et al., 1997). In this approach, the waste activated sludge is collected and directly used in the *accumulation* step for the production of PHA (Arcos-Hernandez et al., 2013). In the *accumulation* reactor, similar conditions of nutrients limitation are used to maximize the cellular PHA content of the PHA-storing biomass. However, the reported maximum achieved PHA contents and yields on substrate with waste activated sludge have been lower on average than those obtained when a PHA producing enrichment culture is used (Kourmentza et al., 2017). Overall, the *enrichment* and the *direct* approaches have been successfully applied in pilot-scale studies and most recently the first demonstration plant was inaugurated into operations in the Netherlands in May 2022 as part of the PHA2USE project.

## 1.5. Scope and outline of this thesis

The aim of this thesis was to study and understand principles that could be applied towards optimized methods for PHA production by directly using waste activated sludge produced in municipal wastewater treatment.

In **Chapter 2**, microbial community-based PHA production at pilot-scale was reviewed and the current knowledge gaps were identified. From this analysis, research questions were proposed and investigated in chapters 3 to 6. Direct comparison between PHA accumulation experiences was challenging, and even contradicting results are regularly reported. This is partly caused by a lack of standardization of PHA accumulation experiments. Consequently, **Chapter 3** focuses on

the influence of different process conditions on the production of PHA, biomass growth and polymer molecular weight. A protocol for standardized PHA accumulation tests was developed based on the outcomes of Chapter 3. In **Chapter 4**, this standardized protocol was applied to activated sludge from six different WWTPs in combination with selective microscope staining to quantify a) the maximum PHA accumulation capacity and b) the relative level of PHA storing bacteria in activated sludge. These tests allowed to estimate the practical limits of PHA content that will likely be feasible to achieve municipal activated sludge. In order to reach the highest practical limits, a strategy based on the selective growth of the PHA-storing biomass during the PHA accumulation process was proposed in **Chapter 5**. Ammonium was required to drive this selective biomass growth and consequently, the activity of ammonium consuming flanking microbial populations, such us nitrifying bacteria, needs to be minimized. In **Chapter 6**, the use of low dissolved oxygen concentrations was evaluated as a means to minimize the activity of nitrifying bacteria. Finally, in **Chapter 7** the key findings of the thesis research are discussed and insights on the future of microbial community-based PHA production are given.

*To Chapter 6*

# 2

# Scaling-up microbial community-based PHA production: status and challenges

A modified version of this chapter has been published as **Estévez-Alonso, Á.**[1], Pei, R.[1], van Loosdrecht, M.C.M., Kleerebezem, R., Werker, A., 2021. *Scaling-up microbial community-based polyhydroxyalkanoate production: status and challenges.* Bioresource Technology, **327**, 124790. [1]Á. Estévez-Alonso and R. Pei contributed equally.

## Abstract

*Conversion of organic waste and wastewater to polyhydroxyalkanoates (PHAs) offers a potential to recover resources from organic waste. Microbial community-based PHA production systems have been successfully applied in the last decade at lab- and pilot-scales, with a total of 19 pilot installations reported in literature. In this review, research at pilot-scale on microbial community-based PHA production is categorized and subsequently analyzed with focus on feedstocks, enrichment strategies, PHA yields on substrate, biomass PHA contents and polymer characterization. From this assessment, the challenges for further scaling-up of microbial community-based PHA production are identified and two application case studies are provided.*

## 2.1. Introduction

Nowadays, as society motivates goals of developing circular economies, converting waste into valuable raw materials is drawing increasing attention (Kehrein et al., 2020). For example, in 2018 every person in The Netherlands produced an average of 87 kg of organic fraction of municipal solid waste (OFMSW), 104 $m^3$ of wastewater and 18 kg of dry sewage sludge waste (CBS (Centraal Bureau voor de Statistiek), 2020a,b). Most of the OFMSW is currently either used for the production of methane containing biogas or compost. The wastewater from households is commonly treated in municipal wastewater treatment plants (WWTP) which discharge treated water but also a significant mass of waste activated sludge (WAS). The waste activated sludge is typically incinerated (with or without pretreatment by anaerobic digestion) and remaining ashes are landfilled (CBS (Centraal Bureau voor de Statistiek), 2020a). In keeping with the new circular economy package proposed by the EU for 2030, there should be well-defined steps undertaken to further develop the end of waste criteria for different waste streams (Commission, 2020). Therefore, from both legislative and environmental sustainability perspectives, the current waste treatment schemes are challenged to become further expanded into a wider repertoire of products and services from the resources that can be recovered from these waste streams. One promising waste valorization route is to produce biopolymers such as Kaumera gum (extracted from aerobic granular sludge) and/or polyhydroxyalkanoates (PHAs) from waste/wastewater organic matter (Feng et al., 2021; Rodriguez Perez et al., 2018). For instance, in 2020 the first full-scale Kaumera gum installation has been launched in Zutphen (The Netherlands). In parallel, microbial community-based PHA production is also moving forward in developments motivating investments for scaling-up.

PHAs are a family of biodegradable polyesters that are naturally synthesized by a wide variety of microorganisms as energy and carbon reserves (Dawes and Senior, 1973). Due to an ecological role as storage polymers, PHAs are usually produced under growth limiting conditions and/or in dynamic environments characterized by the alternating presence and absence of carbon source and/or electron acceptor (Van Loosdrecht et al., 1997; Majone et al., 1999; Reis et al., 2003). PHAs are not soluble in water, therefore they are accumulated by bacteria as cytoplasmic

intracellular granules forming inclusion bodies (Jendrossek and Pfeiffer, 2014). As polymers, PHAs offer promise in a wide variety of applications e.g. packaging, disposable items and/or biodegradable carriers (Raza et al., 2018).

Currently, PHAs are industrially produced by defined bacterial strains, known as pure cultures, that can intracellularly accumulate PHAs up to 90% of their cell dry weight (Koller, 2018). Using pure culture fermentations methods, it is estimated that 25.3 kt PHA are produced yearly (Bioplastics, 2020; Vandi et al., 2018). In a pure culture process, high purity substrates and sterile conditions are required to avoid contamination by non-PHA accumulating microorganisms. The combination of refined substrates and white biotechnology methods for commercial production results in reported high production costs for PHA compared to petroleum-based polymers used within the plastics industry (Raza et al., 2018). Unlike pure cultures, environmental biotechnology aims at designing and engineering process environments rather than working with specific microorganisms and axenic white biotechnology methods (Kleerebezem and van Loosdrecht, 2007). With this in mind, PHA-accumulating bacteria can be enriched by applying selective process conditions that favour PHA-producing microorganisms over non-PHA accumulating microorganisms (Kourmentza et al., 2017). By applying an alternating presence and absence of the carbon source, microorganisms that are able to accumulate PHAs when carbon is present (feast) increase in relative proportions due to a survival advantage during the longer periods without substrate (famine). In such a microbial community-based process, feedstock sterilization for limiting the growth of the non PHA-storing phenotype is not important and therefore common waste streams can be used as feedstock without onerous steps of pre-treatment. Waste streams are normally fermented to produce volatile fatty acids (VFAs) that are subsequently metabolized by suitably enriched cultures to produce PHAs. Microbial community-based PHA production at laboratory scale can achieve a comparable cellular PHA content, as those that have been obtained with pure culture methods (up to 90% cell dry weight) (Johnson et al., 2009a).

After the success of microbial community-based PHA production at lab-scale, research efforts continued with focus on the study of different selective conditions for enrichment of PHA-accumulating microorganisms and with the objective to

maximize the cellular PHA content (Dias et al., 2006; Verlinden et al., 2007; Chanprateep, 2010; Valentino et al., 2017; Kourmentza et al., 2017). In parallel, over the past 10 years efforts to scaling-up the technology have resulted in a total of 19 publications describing successes with pilot-scale experiences in which various waste streams have been used as feedstocks for microbial community-based PHA production and recovery. Despite these successful pilot plant operations to date, breakthrough with a demonstration of commercial full-scale production is still not realized.

This work aims to review the current status of microbial community-based PHA production with emphasis collective experience gained from work at pilot-scale and, from those experiences, identify possible bottlenecks that present challenges to come on the road to realize commercial-scale production of PHAs from waste and wastewater organic matter. For more information about PHA production in general, by pure culture methods and/or in the use of different selection pressures to enrich for a PHA-accumulating biomass, other recently published review articles are available (Kourmentza et al., 2017; Rodriguez Perez et al., 2018; Dietrich et al., 2017; Sabapathy et al., 2020; Li and Wilkins, 2020).

## 2.2. Pilot-scale PHA production

Nineteen published pilot-scale studies on microbial community-based PHA production can be broadly categorized into two different approaches based on biomass source: *enrichment accumulation* and *direct accumulation* approaches, as illustrated in Figure 2.1.

The *enrichment accumulation* approach primarily focuses on the maximization of the PHA production through applied optimum selective pressures. This approach consists of an enrichment/selective step to produce as highly functional biomass as possible for PHA accumulation. This enrichment/selective step has been typically performed in a sequential batch reactor by feeding a pre-fermented VFA-rich stream under the aerobic feast-famine regime (Chakravarty et al., 2010; Tamis et al., 2014a, 2018; Valentino et al., 2018, 2019a,b; Moretto et al., 2020b; Mulders et al., 2020a). After the functional biomass is produced, the same pre-fermented VFA-rich stream is most often used with appropriate modulation of nu-

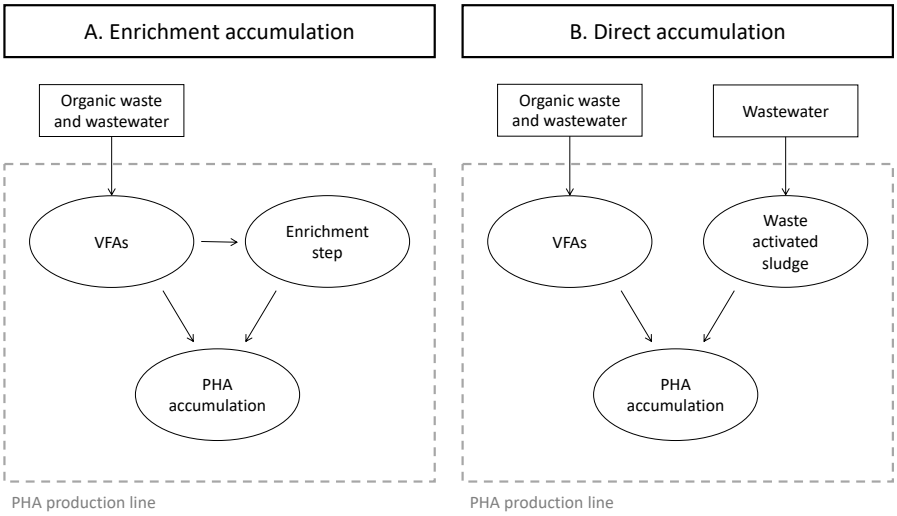


Figure 2.1. Critical defining differences between the *enrichment accumulation* and the *direct accumulation* approaches. Adapted from Bengtsson et al. (2017a).

trient concentrations and under fully aerobic conditions to maximize the biomass PHA content in the PHA accumulation process.

In contrast to *enrichment accumulation*, the *direct accumulation* approach primarily focuses on the use of the PHA-storage capacity of the waste activated sludge for PHA production. This approach is based on the subsequent exploitation of waste activated sludge produced as a by-product from treating wastewater in a WWTP. A waste activated sludge with significant PHA accumulation potential can be found in both industrial or municipal biological WWTP, even though the biological processes were primarily designed for organic carbon and nutrients removal. Municipal or industrial wastewater may not always have significant VFA content, but bioprocess conditions of the WWTP may nevertheless tend to enrich over time for the PHA-storing phenotype (Anterrieu et al., 2014; Morgan-Sagastume et al., 2014, 2015; Bengtsson et al., 2017a,b; Larriba et al., 2020; Conca et al., 2020). Conditions of dynamic substrate supply in these installations, are mainly due to the configuration/flow pattern, creating an inherent selection pressure (Van Loosdrecht et al., 1997). The use of waste activated sludge gives the opportunity to integrate PHA production processes into the municipal wastewater treatment as an extension to present day sludge management goals (Bengtsson et al., 2017b).

However, other supplies of (waste) organic feedstock are required in order to be able to exploit the polymer storing potential of the waste activated sludge exported from the WWTP.



### 2.2.1. Feedstocks

The cost of refined feedstocks for a PHA production process can represent up to 48% of the total costs (Rodriguez Perez et al., 2018). Waste streams as feedstock alongside microbial community-based bioprocess methods is an anticipated means to improve the overall PHA production economy at commercial scale. Moreover, the use of waste streams for PHA production fulfills goals in waste valorization objectives. However, waste streams may bring other complexities for production and product quality control, and especially if the accumulating biomass cannot be acclimated to specific production conditions or feedstock variabilities are too large. Potential challenges of PHA production from waste streams include potential for batch or seasonal compositional variations, too low relative VFA contents (0.35-1.00 $gCOD_{VFA}/gsCOD$), presence of nutrients (100-800 $mgNH_4/L$), high salinity (>5 gNaCl/L), high solids contents (up to 1.5 gTS/L) and other unknown compounds (Table 2.2). Nutrients and high salinity have been found to negatively affect PHA production (Johnson et al., 2009a; Palmeiro-Sánchez et al., 2016a). The non-VFA fraction can promote the growth of non-PHA accumulating biomass and together with high solids contents may act to effectively reduce/dilute the final biomass PHA content (Korkakaki et al., 2016). Unknown compounds could be carried over with recovery and this may negatively affect the polymer physical-chemical quality or its application under selected regulatory frameworks (Laycock et al., 2013).

First experiences at pilot-scale for both *enrichment accumulation* and *direct accumulation* approaches were performed with relatively simple wastewater streams characterized by high VFA contents and low nutrients, solids and salts concentrations i.e. fermented dairy wastewater (Chakravarty et al., 2010), fermented beet process wastewater (Anterrieu et al., 2014), fermented candy factory wastewater (Tamis et al., 2014a; Bengtsson et al., 2017b), fermented paper mill wastewater (Tamis et al., 2018) and starch-rich wastewater (Morgan-Sagastume

Table 2.1. Definitions of the process properties used in this work.

| Property | Description | Units |
|---|---|---|
| Feedstock | Substrate used for PHA production | - |
| Yield on substrate | Fraction of substrate used for PHA production | gCOD/gCOD |
| Biomass PHA content | Ratio between PHA and volatile suspended solids | gPHA/gVSS |
| Polymer properties | | |
| HB content | Fraction of *hydroxybutyrate* in the PHA co-polymer | gHB/gPHA |
| HV content | Fraction of *hydroxyvalerate* in the PHA co-polymer | gHV/gPHA |
| Molecular weight | Molecular weight of the PHA co-polymer | kDa |
| Impurities | Non PHA fraction in the extracted PHA co-polymer | - |

et al., 2020). In more recent years, more complex waste streams have also been successfully evaluated. These streams were characterized by high non-VFA COD composition, excess nutrients, high suspended solids and unknown compounds concentrations i.e. fermented leachate from the OFMSW (Valentino et al., 2018, 2019a,b; Moretto et al., 2020b; Mulders et al., 2020a; Valentino et al., 2020; Moretto et al., 2020a), fermented tomato waste (Bengtsson et al., 2017a) and fermented primary sludge (Morgan-Sagastume et al., 2015; Bengtsson et al., 2017b; Conca et al., 2020). To cope with suspended solids, a solid-liquid separation stage for the feedstock prior entering the PHA production line has been evaluated (Valentino et al., 2018, 2019b; Moretto et al., 2020b; Valentino et al., 2020; Moretto et al., 2020a). Suspended solids for PHA accumulation feedstocks after fermentation were reduced to levels in the order of 0.02 gTSS/gsCOD by drum filtration (Bengtsson et al., 2017b). To deal with the excess of growth nutrients that may promote the growth of non PHA-accumulating microorganisms, phosphorus levels were reduced to a COD:P of 100:0.1 (mass basis) by iron chloride precipitation before entering the PHA line (Bengtsson et al., 2017b). Alternatively, a settling step, in the middle of the enrichment cycle, was used to limit the growth of non PHA-accumulating microorganisms by removing the non-VFA COD fraction, right after the VFA-COD fraction was consumed (Mulders et al., 2020a).

One can conclude that it is technically feasible to produce PHAs from different

Table 2.2. Feedstocks used for the PHA accumulation reactors.

| Feedstock | TS | VS | sCOD | VFA/sCOD | C2 | C3 | C4 | C5 | $NH_4^+$ | $PO_4^-$ | Alkalinity | pH | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | gTS/L | gVS/L | gCOD/L | - | % | % | % | % | mgN/L | mgP/L | $mgCaCO_3/L$ | - | |
| Acetic acid | n.a. | n.a. | 172.0 | 100 | 100 | 0 | 0 | 0 | 0 | 1541 | - | - | (Patel et al., 2009) |
| Acetic/Propionic acid | n.a. | n.a. | 86.0 | 100 | - | - | - | - | 0 | 1541 | - | - | |
| Fermented dairy wastewater | - | - | 2.9-3.2 | - | - | - | - | - | - | - | - | 6 ± 0.3 | (Chakravarty et al., 2010) |
| Fermented beet process water | - | - | 9.9 ± 1.3 | 35-90 | 28 | 33 | 39 | - | 147 ± 4 | 23 ± 5 | - | - | (Anterrieu et al., 2014) |
| Acetic acid | n.a. | n.a. | - | 100 | 100 | 0 | 0 | 0 | 0 | 0 | - | - | (Morgan-Sagastume et al., 2014) |
| Fermented candy factory wastewater | - | - | 7.8 ± 4.1 | 64 ± 15 | 32 | 14 | 33 | 5 | Residual | Residual | - | 4.5 ± 0.1 | (Tamis et al., 2014a) |
| Primary sludge centrate | - | - | 9.0 ± 1.0 | 90 ± 9 | 76 | 24 | 0 | 0 | 900 ± 100 | 480 ± 100 | - | 5.6 -6.4 | (Morgan-Sagastume et al., 2015) |
| Acetic acid | n.a. | n.a. | - | 100 | 100 | 0 | 0 | 0 | Growth-limiting and excess | | - | - | |
| Tomate waste centrate | - | - | 9.7-12.4 | 80-86 | 34 | 23 | 17 | 16 | 174-223 | 58-74 | - | 5.6 | (Bengtsson et al., 2017a) |
| Acetic acid | n.a. | n.a. | 80 | 100 | 100 | 0 | 0 | 0 | 800 | 40 | - | 4.5 | |
| Synthetic mix | n.a. | n.a. | 10 | 100 | 85-99 | 1-15 | 0 | 0 | 100 | 5 | - | 5 | (Bengtsson et al., 2017b) |
| Fermented candy factory wastewater | 0.2 | - | 16 | - | C2+C4: 60-95 | | C3+C5: 5-40 | | 80 | 16 | - | 5.5-6.0 | |
| Primary sludge centrate | - | - | 7 | - | C2+C4: 50-75 | | C3+C5: 25-50 | | 350 | 7 | - | 4.8-5.5 | |
| Fermented paper mill wastewater | - | - | ≈6.2 | 72 | 37 | 21 | 29 | 16 | Residual | Residual | - | 5.0 | (Tamis et al., 2018) |
| Acetic acid | n.a. | n.a. | - | 100 | 100 | 0 | 0 | 0 | 0 | 0 | - | - | (Valentino et al., 2018) |
| Fermented OFMSW | n.d. | n.d. | 16.0 ± 0.7 | 91 ± 9 | 21 | 13 | 38 | 12 | 400-480 | 80-112 | 894 ± 104 | 5.0 ± 0.2 | |

*meq/L; n.a.: not applicable; n.d. not detectable.

Continuation of Table 2.2.

| Feedstock | TS<br>gTS/L | VS<br>gVS/L | sCOD<br>gCOD/L | VFA/sCOD<br>- | C2<br>% | C3<br>% | C4<br>% | C5<br>% | $NH_4^+$<br>mgN/L | $PO_4^-$<br>mgP/L | Alkalinity<br>$mgCaCO_3/L$ | pH<br>- | References |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fermented OFMSW and SAS mix | n.d. | n.d. | 16.2 ± 0.5 | 90 ± 2 | 23 | 13 | 37 | 11 | - | - | 2800 ± 200 | 5.0-5.5 | (Valentino et al., 2019a) |
| Acetic acid | n.a. | n.a. | - | 100 | 100 | 0 | 0 | 0 | 0 | 0 | - | - | |
| Fermented OFMSW and SAS mix I | n.d. | n.d. | 32 ± 5 | 64 ± 7 | 28 | - | 28 | - | 724 ± 138 | 127 ± 22 | 4811 ± 741 | 5.0-5.5 | (Valentino et al., 2019b) |
| Fermented OFMSW and SAS mix II | n.d. | n.d. | 26 ± 3 | 75 ± 9 | 33 | - | 23 | - | 562 ± 44 | 110 ± 9 | 4451 ± 498 | 5.0-5.5 | |
| Fermented OFMSW and SAS mix | n.d. | n.d. | 34 ± 3 | 86 ± 5 | C2+C4: 75 | | C3+C5: 25 | | 570-873 | 130-152 | - | 5.0-5.5 | (Moretto et al., 2020b) |
| Municipal wastewater | - | - | 0.14 ± 0.04 | - | - | - | - | - | 40± 11 | 4.2 ± 1.1 | - | - | (Larriba et al., 2020) |
| Acetic acid | n.a. | n.a. | - | 100 | 100 | 0 | 0 | 0 | 0 | 0 | - | - | |
| Fermented cellulosic primary sludge liquid | - | - | 8.8 ± 1.6 | 94 | 24 | 50 | 12 | 9 | 326 ± 23 | 70 ± 12 | - | 4.8 ± 0.1 | (Conca et al., 2020) |
| Fermented OFMSW | 1.5 ± 0.8 | 0.9 ± 0.5 | 5.8 ± 1.1 | 50 ± 13 | C2+C4: 56 | | C3+C5: 44 | | 622 ± 159 | 20.9 ± 10.8 | 70 ± 10* | 7.5 ± 0.4 | (Mulders et al., 2020a) |
| Fermented OFMSW | n.d. | n.d. | 20 ± 3 | 73 ± 8 | - | - | - | - | 615 ± 37 | 140 ± 13 | - | - | |
| Fermented OFMSW and SAS mix | n.d. | n.d. | 27 ± 4 | 85 ± 9 | - | - | - | - | 673 ± 72 | 119 ± 11 | - | - | (Valentino et al., 2020) |
| Fermented OFMSW and SAS mix | n.d. | n.d. | 36 ± 2 | 86 ± 5 | C2+C4: 54-74 | | C3+C5: 35-55 | | 689 ± 15 | 220 ± 6 | - | 5.0-5.5 | (Moretto et al., 2020a) |
| Acetic acid | n.a. | n.a. | 9.0 | 100 | 100 | - | - | - | 90 | 4.5 | - | - | |
| Acetic/propionic acid | n.a. | n.a. | 9-100 | 100 | C2+C4: 60-100 | | C3+C5: 0-40 | | 1000 | 50 | - | 5.6 | (Morgan-Sagastume et al., 2020) |
| Potato-starch factory effluent | 0.3 ± 0.1 | 0.2 ± 0.1 | 9.5 ± 1.6 | 73 ± 10 | 94 | - | - | - | n.d. | 18 ± 56 | - | 4.8-6.5 | |

*meq/L; n.a.: not applicable; n.d. not detectable.

waste streams, either with biomass produced through specialized enrichment processes or produced as a by-product of mainstream biological wastewater treatment. Further up-scaling of the microbial community-based PHA production requires that at least one potential feedstock specifically for the polymer production is selected. The selected feedstock must be such that the volume of biomass and feedstock supplies are relevant towards meeting the demand in amount and quality of polymers supply in suitable economically supporting commercial application(s). Even if technical feasibility has been established for diverse feedstocks at pilot scale, evaluation and understanding of the relevance of the feedstocks in the context of a commercial production scale is still lacking in the literature.

### 2.2.2. Yields of PHA on substrate

Yields on substrates are defined as the amount of PHA produced per gram of consumed VFA ($gCOD_{PHA}/gCOD_{VFA}$) or per gram of consumed waste (gPHA/gWaste). However, how yields on substrate are reported in current literature can be misleading if applied with direct comparison. Many authors have reported the average yields when the PHA content reached saturation levels, while others reported the average yield when 95% of the maximum PHA content was reached (Rodriguez Perez et al., 2018). Similarly, some authors referred to the PHA yield on VFA while others have evaluated the yield on soluble COD. A common basis for description of the process yield is necessary towards making meaningful insights of process or feedstock related differences. As Rodriguez Perez et al. (2018) proposed, it is recommended at least to report both PHA yield related to the added VFA and PHA yield related to the added waste (on a COD basis). Yield should be calculated for the same relative time points in the PHA accumulation process. It is considered that this time point should be relative to degree of saturation, rather than an absolute point in time since kinetics of accumulation may differ even for the same biomass source (Morgan-Sagastume et al., 2020).

Ideally, the yields on substrates should be as close to the maximum theoretical values and as consistent as possible from production batch to batch. The PHA yields on substrate are considered to be affected by the presence or absence of nutrients (N and P) and the VFA composition (Shi et al., 1997). PHA production is

usually seen as an overflow mechanism in which PHA is produced when the specific substrate uptake rate exceeds the substrate flux that is used for growth (Tamis et al., 2014a). Therefore, if nutrients are limiting in the feedstock, growth can be minimized and PHA production would be maximized (Johnson et al., 2009a). Moreover, the maximum theoretical yield is only determined by the feedstock composition. Butyrate and valerate have slightly higher COD-based product yields compared to acetate and propionate. This difference can be explained by the fact that these polymer precursors (butyrate and valerate) require less ATP per unit of COD to be converted into PHA than the others (acetate and propionate). Consequently, the theoretical PHB yield on butyrate is 0.84 $gCOD_{PHB}/gCOD_{Butyrate}$ compared to 0.75 $gCOD_{PHB}/gCOD_{Acetate}$ on acetate (Marang et al., 2013). Differences in reported yield between pilot installations may come from the feedstock composition and not only from how the system was operated. Therefore, it is recommended to always report a full description of the VFAs present in the feedstock for meaningful research contribution. Additionally, future work on the fermentation of waste streams prior to the PHA production line can focus on methods for selectively producing more butyrate and valerate rather than acetate and propionate.

Regarding the *enrichment accumulation* approach, reported average yields in the accumulation reactor are found to be similar and close to the theoretical maximum. Yields of 0.61-0.68 $gCOD_{PHA}/gCOD_{VFA}$ were observed when synthetic or nutrient-poor streams were used as feedstocks (Tamis et al., 2014a, 2018; Valentino et al., 2019b, 2018; Conca et al., 2020). However, when nutrients-rich streams were applied, a wider range with lower average yields have been reported, 0.33-0.61 $gCOD_{PHA}/gCOD_{VFA}$ (Valentino et al., 2018, 2019b; Moretto et al., 2020b; Valentino et al., 2020; Moretto et al., 2020a). This was not the case for Mulders et al. (2020a) that reported the highest PHA accumulation average yield on substrate so far at pilot-scale, 0.73 $gCOD_{PHA}/gCOD_{VFA}$ while working with leachate from OFMSW, a nutrients-rich and acetate-rich waste stream (0.44 ± 0.11 $gCOD_{Acetate}/gCOD_{VFA}$). In the *enrichment accumulation* approach, 25-50% of the feedstock is directed to biomass production in the enrichment reactor. Effectively, the overall average PHA yield on substrate is lower, ranging between 0.17-0.55 $gCOD_{PHA}/gCOD_{VFA}$. Consistent yields are reported within the same work (<10% error), however large differ-

ences between different pilot scale works seem to exist. The reason for such case to case variability is unclear and this suggests need for deepened fundamental insight concerning regulating factors of process or context.

In the *direct accumulation* approach, the reported average accumulation yields have generally been lower and more variable in nature, independently of the nutrients levels and composition of the feedstock, 0.20-0.61 $gCOD_{PHA}/gCOD_{VFA}$ (Anterrieu et al., 2014; Morgan-Sagastume et al., 2015; Bengtsson et al., 2017a,b; Conca et al., 2020; Morgan-Sagastume et al., 2020). An acclimation to the feedstock has been reported to improve the performance of direct accumulation (Morgan-Sagastume et al., 2019). It is important to emphasize that in the *direct accumulation* approach the feedstock is used only for PHA synthesis in the accumulation reactor, in contrast to the *enrichment accumulation* approach. Thus, the overall performance of PHA production yield is similar (neglecting biomass production as a by-product of services in waste water treatment).

Independently of the approach used, it was observed that the PHA average yield on substrate decreased over the time of accumulation, when feedstocks with growth nutrients are used. It was, therefore, suggested to keep the accumulation periods as short as needed to reach PHA saturation levels (Bengtsson et al., 2017b; Tamis et al., 2018). In order to keep high average PHA yields in longer accumulation runs, Valentino et al. (2015) suggested to incorporate simultaneous growth of the PHA-accumulating microorganisms in the PHA accumulation run. Daughter cells have been observed to contain half of the cellular PHA content of the mother cells (Pfeiffer and Jendrossek, 2012). As new cells (with more available space for PHA) are produced, PHA production rates can be maintained high for longer periods of time even if more substrate is directed away from conversion to PHA. Mechanisms to favour the growth of PHA-accumulating microorganisms over non PHA-accumulating microorganisms do not appear to be well-established in the research literature and these methods remain to be further developed. A combination of nutrient levels and a feed-on-demand feeding strategy were shown to promote the growth of only PHA-accumulating microorganisms during the PHA accumulation without loss of overall biomass PHA content (Valentino et al., 2015; Mulders et al., 2020b). Sustained selective growth under conditions of partial phos-

phorus limitation has been applied as a biomass enrichment method starting with municipal activated sludge (Cavaillé et al., 2013).

The feedstock complexity, the presence of nutrients and different salinity levels can be factors limiting the potential level of PHA yields on substrate. However, recent research still suggests a potential to achieve consistent PHA yields from batch to batch, and close to the theoretical maximum, when either the *enrichment* or the *direct* accumulation approaches were used. Overall it can be concluded that specific feedstock dependent pretreatment methods enable high PHA yields. As the PHA yield on soluble COD determines how much substrate eventually ends up in PHA, these results facilitate improvements in performance and economy for the further scaling-up of microbial community-based PHA production.

### 2.2.3. Operational conditions and dominant species

The feedstock composition and the operational conditions of the bioprocess selecting for growth of the PHA-storing phenotype determine the microbial community structure in the process and therefore the maximum PHA content that can be achieved per unit of total biomass. It was already shown that the alternating presence of carbon source (feast-famine regime) effectively enriches for PHA-accumulating bacteria and this has been the adopted approach for the biomass production explicitly (*enrichment accumulation*) or implicitly (*direct accumulation*) in all the reported research work performed at pilot-scale. However, modifications of the operational conditions in the selection reactor have resulted in very different outcomes (Table 2.3). In most of the pilots, pH was only monitored, but not controlled and pH ranged from 6.5 to 9.2. Only in a couple of studies has pH been explicitly controlled between 6.5 and 7.5 (Tamis et al., 2014a, 2018). Hydraulic and solids retention times during biomass enrichment were lower than 2 d, excluding those from the *direct accumulation* approach, in which nutrients removal and sludge production were coupled, and were in the range 5-20 d (Bengtsson et al., 2017b; Conca et al., 2020; Morgan-Sagastume et al., 2020). Temperature was generally not controlled and in the range 15 to 35°C, but when temperature was controlled, the values were 22-25°C, 25-28°C and 30 ± 2°C (Tamis et al., 2014a, 2018; Valentino et al., 2019b; Moretto et al., 2020b,a; Mulders et al., 2020a).

Table 2.3. Operational conditions of the selection reactor at pilot-scale level.

| Feedstock | Enrichment | OLR gCOD/(L d) | HRT d | SRT d | Cycle lenght d | Temperature °C | pH | Dominant Microorganism | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Acetic acid | Aerobic feast Aerobic famine | 0.6 | 1 | 10 | 0.5 | 35 | 8.3-8.7 | - | (Patel et al., 2009) |
| Acetic acid/propionate | | 0.6 | 1 | 5 | 0.5 | 35 | 8.3-8.7 | - | |
| Fermented dairy wastewater | Aerobic feast Aerobic famine | - | 0.72 | 2.96 ± 2 | - | - | 8.2 ± 0.2 | - | (Chakravarty et al., 2010) |
| Fermented beet process wastewater | Aerobic feast Aerobic famine | - | 5 | - | - | - | - | - | (Anterrieu et al., 2014) |
| Municipal wastewater | Aerobic feast Aerobic famine | 3 | 0.21 | 1-2 | 0.08 | - | - | - | (Morgan-Sagastume et al., 2014) |
| Fermented candy factory wastewater | Aerobic feast Aerobic famine | ≈5 | 1 | 1 | 0.5 | 30 ± 2 | 6.5-7.5 | P. acidivorans | (Tamis et al., 2014a) |
| Municipal wastewater | Anoxic feast Aerobic famine | 3.0 ± 0.8 | 0.125 | | 1.7 ± 1.2 | 8.4-22.8 | - | - | (Morgan-Sagastume et al., 2015) |
| Tomate waste centrate | Anoxic feast Aerobic famine | 1.8 ± 0.7 | 0.125-0.25 | 5.9 | 0.08 | 20 | - | - | (Bengtsson et al., 2017a) |
| Municipal wastewater | Anoxic feast Aerobic famine | - | - | 17 | - | 10-23 | - | - | (Bengtsson et al., 2017b) |
| Fermented paper mill wastewater | Aerobic feast Aerobic famine | ≈5 | 1 | 1 | 0.5 | 30 ± 2 | 6.6-7.2 | P. acidivorans | (Tamis et al., 2018) |
| Fermented OFMSW | Aerobic feast Aerobic famine | 2.5-3.0 | 1 | 1 | 0.25 | 14-29 | 8.0-8.5 | - | (Valentino et al., 2018) |

Continuation of Table 2.3.

| Feedstock | Enrichment | OLR gCOD/(L d) | HRT d | SRT d | Cycle lenght d | Temperature °C | pH | Dominant Microorganism | Reference |
|---|---|---|---|---|---|---|---|---|---|
| Fermented OFMSW | Aerobic feast Aerobic famine | 2.0-3.4 | 1 | 1 | 0.25 | 16-28 | 8.0-8.7 | Hydrogenophaga spp | (Valentino et al., 2019a) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | 4.0 | 1 | 1 | 0.25 | 22-25 | 8.0-9.0 | Hydrogenophaga spp | (Valentino et al., 2019b) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | 3.1-5.9 | 1 | 1 | 0.25 | 25-28 | - | Hydrogenophaga spp | (Moretto et al., 2020b) |
| Municipal wastewater | Anaerobic feast Anoxic famine | - | 1.0-1.5 | 10-15 | 0.3-0.5 | - | - | - | (Larriba et al., 2020) |
| Fermented cellulosic primary sludge liquid | Aerobic feast Anoxic famine | 0.89-1.58 | 1.7-2.3 | 6-7 | ≈0.5 | 18.8-26.8 | - | - | (Conca et al., 2020) |
| Fermented OFMSW | Aerobic feast Aerobic famine | ≈8 | 0.71 | 1 | 0.5 | 30±3 | 8.5-9.2 | Uncultured Rhodocyclaceae | (Mulders et al., 2020a) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | 2.2-4.4 | 1 | 1 | 0.25 | 15-34 | 8.0-9.0 | - | (Valentino et al., 2020) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | 4.0 | 1-2 | 1-2 | 0.25-0.5 | 25-28 | - | - | (Moretto et al., 2020a) |
| Potato-starch factory effluent | Aerobic feast Aerobic famine | 2.2 ± 0.4 | 4.5 ± 0.6 | 7.1 ± 1.1 | 0.33 | 27 ± 3 | 8.5 ± 0.4 | - | (Morgan-Sagastume et al., 2020) |

Notwithstanding, selection has been suggested to be sensitive to temperature and pH based on studies where the resultant dominant microorganisms were assessed under the applied selection conditions. For the *enrichment accumulation* approach, under well-defined conditions of temperature and pH (pH 7 and 30°C), *Plasticicumulans acidivorans* was found to be the dominant microorganism (Tamis et al., 2014a, 2018). When temperature remained at 30°C, but pH was not controlled, and resulted in pH ranging between 8.5 and 9.2, an uncultured *Rhodocyclaceae* bacterium clone JT01 was found to be the dominant microorganism (Mulders et al., 2020a). Under similar pH conditions, but lower temperature (15-29°C) a member of the *Hydrogenophaga* spp. were enriched (Crognale et al., 2019). These outcomes emphasize that changes in operational conditions, such as pH or temperature, may result in different dominant microorganisms (Crognale et al., 2019; Mulders et al., 2020a), which may ultimately be an important factor that influences the maximum PHA content that can be achieved in an accumulation process. Temperature was found to be optimum at 30°C in lab-scale systems for the enrichment of high PHA-accumulating microorganisms (Stouten et al., 2019). However, reasons why different dominant species are enriched under apparently similar conditions remains an open question begging deepened understanding.

In the *direct accumulation* strategy, there has been already a lot studied about the microbial structure of waste activated sludge (Cydzik-Kwiatkowska and Zielińska, 2016). However only a limited focus has been given in different studies on the abundance of the PHA production phenotype in waste activated sludge samples (Morgan-Sagastume, 2016). It has been observed that the PHA accumulation potential of biomass produced in enhanced biological phosphorus removal processes (bio-P sludge) was lower than those from biomass enriched under aerobic and/or anoxic conditions (Bengtsson et al., 2017b). Fundamental understanding about why bio-P activated sludge has shown less PHA accumulation potential compared to non bio-P activated sludge is still unknown and also remains a point of misunderstanding especially in the municipal sector given the common popular association of bio-P metabolism with PHA storage.

One of the main differences between the *enrichment accumulation* and the *di-*

*rect accumulation* approach is the process operational conditions. Differences in operational conditions have also resulted in different dominant species with different characteristics. The fundamental reason of why PHA accumulating microorganisms with high maximal PHA contents are enriched instead of PHA accumulating microorganisms with low maximal PHA contents with similar feedstocks, feast-famine cycle lengths, and temperature requires further investigation.

### 2.2.4. Biomass PHA content

Biomass PHA content, in most cases, refers to a relative amount of PHA and with respect to the biomass volatile and/or total suspended solids (gPHA/gVSS or gPHA/gTSS). The reported maximum attainable amounts of biomass PHA content is one of the major differences in expectation between *enrichment accumulation* and *direct accumulation* approaches. Processes of *enrichment accumulation* are typically tuned to be able to produce a more specialized biomass for PHA production, and as such, with expected higher PHA accumulation potential. Biomass PHA content is important because it can influence on the costs of the downstream processing (DSP) and the recovered polymer quality. To recover the same mass of recovered PHA, greater amounts of biomass need to be processed the lower the biomass PHA contents.

With the *enrichment accumulation* approach, high PHA contents, 0.7-0.8 gPHA/gVSS, were attained in pilot accumulations with pre-fermented industrial wastewater streams from candy and paper mill factories (Tamis et al., 2014a, 2018). With similar process configuration but without pH control Mulders et al. (2020a) reported 0.77 gPHA/gVSS by using fermented OFMSW as feedstock. Using fermented OFMSW or a filtered fermented mixture of 30% OFMSW and 70% biological sludge (v/v), a series of accumulation batches PHA contents in the PHA-rich biomass ranged between 0.33 gPHA/gVSS and 0.59 gPHA/gVSS (Valentino et al., 2018, 2019a,b; Moretto et al., 2020b; Valentino et al., 2020; Moretto et al., 2020a).

Using the *direct accumulation* approach, accumulation up to 0.52 gPHA/gVSS were reported when feeding fermented waste VFA-rich streams to waste activated sludge from selected municipal and/or industrial WWTPs (Bengtsson et al., 2017b; Conca et al., 2020; Morgan-Sagastume et al., 2020). These results were obtained

without any modification to the wastewater treatment line, even if simple adjustment or process modifications were foreseen to introduce improvements due to imposed periodic feast stimulation to the process biomass. It was interpreted that the quality of the ”feast” environment established in the full-scale process was influential to the waste activated sludge capacity for PHA storage. To demonstrate a potential to engineer selective pressure for the PHA storing phenotype while treating the municipal wastewater, biomass was produced based on a pilot-scale anoxic-feast and aerobic-famine selection pressure (Bengtsson et al., 2017b). The waste activated sludge from the pilot system was found to accumulate up to 0.49 gPHA/gVSS compared to the waste activated sludge coming from the full-scale installation that could only accumulate up to 0.15 gPHA/gVSS. An essential difference in process was the quality of the feast environments between pilot and full-scale systems given the same wastewater.

Pilot-scale PHA contents achieved have been reported in the range of 0.4-0.8 gPHA/gVSS for the *enrichment accumulation* approach and in the range of 0.4-0.6 gPHA/gVSS for the *direct accumulation* approach. Results within individual studies have been relatively robust even if outcomes in accumulation potential are varied between the respective piloting experiences. Reasons why outcomes of PHA accumulation potentials are varied between the piloting experiences provided in the literature may be related to:

1. growth of non-storing organisms, diluting the PHA-rich biomass;
2. degree of enrichment with a reduced fraction of PHA producing biomass;
3. production of other storage products;
4. differences in the individual species respective maximum possible PHA content;
5. differences in the physiological state of the PHA-accumulating biomass at the time of accumulation.

Table 2.4. Operational conditions of the accumulation reactor and PHA quality at pilot-scale level.

| Feedstock | Enrichment | Biomass | $Y_{PHA/VFA}$ $gCOD_{PHA}/gCOD_{VFA}$ | PHA Content gPHA/gVSS | HV % | Mw kDa | Effluent | Reference |
|---|---|---|---|---|---|---|---|---|
| Acetic acid<br>Acetic/Propionic acid | Aerobic feast<br>Aerobic famine | Same as enrichment | - | 0.21 ±0.02<br>0.25 ±0.03 | 0<br>5.6 | 2200<br>2300 | Yes | (Patel et al., 2009) |
| Fermented dairy wastewater | Aerobic feast<br>Aerobic famine | Same as enrichment | 0.21-0.26 | 0.39-0.43 | - | - | - | (Chakravarty et al., 2010) |
| Fermented beet process wastewater | Aerobic feast<br>Aerobic famine | Same as enrichment | - | 0.60 | - | - | No | (Anterrieu et al., 2014) |
| Municipal wastewater | Aerobic feast<br>Aerobic famine | Municipal activated sludge | 0.20-0.38 | 0.19-0.34 | 15 | 980 | - | (Morgan-Sagastume et al., 2014) |
| Fermented candy factory wastewater | Aerobic feast<br>Aerobic famine | Same as enrichment | 0.30 ±0.04* | 0.70-0.80 | 16 | - | N,P | (Tamis et al., 2014a) |
| Primary sludge centrate<br>Acetic acid | Anoxic feast<br>Aerobic famine | Municipal activated sludge | 0.25-0.37 | 0.27-0.38<br>0.33-0.39 | 0-30<br>- | 500<br>- | Yes | (Morgan-Sagastume et al., 2015) |
| Tomato waste centrate<br>Acetic acid | Anoxic feast<br>Aerobic famine | Municipal activated sludge | 0.30-0.39<br>0.34-0.53 | 0.34-0.45<br>0.19-0.49 | 42-49<br>0 | - | Yes | (Bengtsson et al., 2017a) |
| Synthetic mixture<br>Fermented candy factory wastewater<br>Primary sludge concentrate | Anoxic feast<br>Aerobic famine | Municipal activated sludge | 0.35-0.48<br>0.28-0.52<br>0.28-0.55 | 0.37-0.43<br>0.37-0.43<br>0.28-0.42 | 0-44 | 700-1500 | - | (Bengtsson et al., 2017b) |
| Fermented paper mill wastewater | Aerobic feast<br>Aerobic famine | Same as enrichment | 0.68 | 0.65-0.76 | 25 | - | No | (Tamis et al., 2018) |
| Fermented OFMSW<br>Acetic acid | Aerobic feast<br>Aerobic famine | Same as enrichment | 0.43-0.57<br>0.61-0.64 | 0.39-0.52<br>0.37-0.42 | 7-13<br>0 | - | Yes | (Valentino et al., 2018) |

Continuation of Table 2.4.

| Feedstock | Enrichment | Biomass | $Y_{PHA/VFA}$ $gCOD_{PHA}/gCOD_{VFA}$ | PHA Content gPHA/gVSS | HV % | Mw kDa | Effluent | Reference |
|---|---|---|---|---|---|---|---|---|
| Fermented OFMSW | Aerobic feast Aerobic famine | Same as enrichment | 0.33-0.44 | 0.38-0.49 | 11-13 | - | - | (Valentino et al., 2019a) |
| Fermented OFMSW and SAS mix I | Aerobic feast Aerobic famine | Same as enrichment | 0.50 ± 0.04 | 0.43±0.01 | 10 | - | - | (Valentino et al., 2019b) |
| Fermented OFMSW and SAS mix II | | | 0.44±0.03 | 0.46±0.05 | 13 | | | |
| Acetic acid | | | 0.67±0.05 | 0.40±0.02 | 0 | | | |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | Same as enrichment | 0.59±0.04 | 0.52±0.04 | - | - | - | (Moretto et al., 2020b) |
| Municipal wastewater | Anaerobic feast Aerobic (Anoxic) famine | Municipal activated sludge | - | 0.03-0.07 | - | - | - | (Larriba et al., 2020) |
| Fermented cellulosic primary sludge liquid | Aerobic feast Anoxic famine | Same as enrichment | 0.61±0.07 | 0.44±0.06 | 21-41 | - | Yes | (Conca et al., 2020) |
| Acetic acid | | | 0.60±0.06 | 0.47±0.05 | 0 | | | |
| Fermented OFMSW | Aerobic feast Aerobic famine | Same as enrichment | 0.44** | 0.77±0.18 | 50 | - | - | (Mulders et al., 2020a) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | Same as enrichment | 0.33-0.47 | 0.36-0.48 | - | - | - | (Valentino et al., 2020) |
| Fermented OFMSW and SAS mix | Aerobic feast Aerobic famine | Same as enrichment | 0.47-0.59 | 0.40-0.59 | - | - | - | (Moretto et al., 2020a) |
| Acetic acid | Aerobic feast Aerobic famine | Same as enrichment | 0.67±0.15 | 0.52±0.05 | 0.1±0.1 | 548±66 | - | (Morgan-Sagastume et al., 2020) |
| Acetic/Propionic acid | | | - | 0.52-0.61 | 24/63 | 181/602 | | |
| Potato-starch factory effluent | | | 0.69±0.15 | 0.45±0.06 | 1.9±0.8 | 547±78 | | |

All these interpretations are plausible in the context of the studies that have been made. However, as mentioned above, the measurement of biomass PHA content to date are ambiguous to understand if and/or when these five factors may apply, more or less. At the same time, these five points also motivate that other kinds of measurements than just PHA content, coupled with continued fundamental investigations are warranted towards improved productivity and control from a greater command in applied methods of environmental biotechnology.

Overall, the enrichment accumulation and direct accumulation strategies have repeatedly achieved biomass PHA content in excess of 0.4 gPHA/gVSS. Above this level, it has been suggested that DSP becomes increasingly more economically viable (Reis et al., 2003). Even though differences in PHA content are found case-to-case, results from piloting experiences nevertheless suggest a maturity in technological feasibility and readiness level to produce polymers by a microbial community based approach.

### 2.2.5. Polymer quality characterization

PHAs as polymers can be characterized in terms of attributes related to copolymer composition and its distribution, average molecular weight (Mw) and its distribution, thermal properties, mechanical properties and impurities (Laycock et al., 2013; Bengtsson et al., 2017b). The polymer properties are not constant through the PHA production process. Different operational conditions in the upstream will lead microorganisms to accumulate PHAs with different physicochemical properties. The evolution of polymer properties during the PHA accumulation process it is not well understood. Importantly, the polymer properties generated in the upstream are interlinked with selection of DSP methods, and the DSP also will further modulate polymer properties. The final PHA properties after DSP determines the range of possible applications for the polymers. From these 19 pilot studies, the polymer properties after the accumulation and before the DSP are the most frequently reported.

Currently, for microbial community-based PHA production, the most common polymers are comprised of a blend of HB (3-hydroxybutyrate) and HV (3-hydroxyva- lerate) monomers. From different pilot studies, the reported HV frac-

tion has varied between 0 to 50%, as described in Table 2.4. The main reason for differences obtained for HB/HV ratios is the feedstock VFA composition (Jiang et al., 2011a). It has been demonstrated that even for a full-scale activated sludge over 4-seasons of operations, polymer type was predictable as a function of the feedstock composition (Werker et al., 2020). Polymer properties were directly related to the average HV content assuming distributions in blends of random co-polymers. Feedstock variability influencing batch-to-batch polymer composition product quality was shown to be controllable by suitable blending of batches into a master batch as part of the polymer recovery and purification. After blending, PHAs can be extracted from this master batch at a larger scale which favours an improved scale in economy for the cost of the extraction processes (Bengtsson et al., 2017b; Werker et al., 2020). More importantly, it was indicated that the HV content of the blended master batch is predictable if the HV contents from the different inputs are known. This predictability gives an opportunity to manipulate the HV content during the DSP for different industrial applications. HV content and its distribution influences crystallization and crystallinity of these co-polymer blends can mean specific requirements for tuning of DSP conditions (Laycock et al., 2013; Koyama and Doi, 1997; Chan et al., 2017; Cal et al., 2019; Werker et al., 2020). It was reported with the same thermal history, the higher the average HV content up to the eutectic point of the co-polymer blend, the lower the maximum crystallinity of the polymer. The maximum crystallinity of the co-polymer blends to be recovered are closely tied to the extraction conditions such as the selection of the type of extraction solvent, the optimum extraction temperature and the duration of the extraction. When it comes to water-based methods of polymer purification, polymer crystallinity influences the survivability of the polymer as a function of time, pH and temperature (Yu et al., 2005; Yu, 2009; Porter and Yu, 2011).

Besides HV content, modulating crystallinity, Mw and thermal stability of recovered PHAs is important with respect to possible methods of formulation and processing alongside targeted properties in the specific context of the intent for the material in application. For example, using PHA for fiber spinning would require higher Mw than using PHA as an additive in other polymers (Bengtsson et al., 2017b). Even though the produced polymer with different Mw would have differ-

ent opportunities in types of possible applications, one still may prefer to produce the polymer with a higher Mw since it will enlarge the window of oportunity for the range of possible applications. However, so far, Mw has been only characterized in few studies and range up to 2300 kDa (Bengtsson et al., 2017b; Patel et al., 2009; Morgan-Sagastume et al., 2014, 2015, 2020). The Mw of the polymer could be affected by the feeding strategy, the presence of alcohols in the upstream process and the drying in the DSP (Werker et al., 2020). The effects of the DSP on Mw and thermal stability are shown to be predictable, nonetheless, similar to the HV content the prediction requires the knowledge of Mw and thermal stability of PHA in the biomass after the accumulation and before the DSP.

As discussed before, biomass PHA content has an impact on the cost of DSP, besides that higher levels of co-extracted non-PHA biomass add complexity to the purification. Additionally, some specific impurities, such as cations, can negatively influence the polymer chemical and/or thermal stability (Csomorova et al., 1994). Carry-over of non-polymer impurities including heavy metals and priority organic pollutants will also influence the scope for application of the polymers due to regulatory frameworks under EU directives or similar (Werker et al., 2020; Astolfi et al., 2020; Riccardi et al., 2020). Thus, the type and amount of specific impurities are critical to consider rather than simply polymer purity for a given method of DSP.

A so-called demonstration scale project using feedstocks intended for full commercial activities will need to address the specifics of opportunities and challenges in the product quality assurance control methods. This is the context where a more detailed polymer characterization can address the most relevant and very case specific knowledge gaps in jumping from current levels of success in findings of technical feasibility to details of process and method for a given waste-to-renewable resource value chain with economic viability including secured supply chains, and well-defined targets of products within a given regulatory framework. A better polymer characterization would be beneficial for developments in both the upstream bioprocesses and downstream purification steps with regards to process and product stability, and this in the end would help towards building of an overall well-functioning value chain.

## 2.3. Challenges for the scaling-up from piloting experience



From the published experiences with microbial community-based PHA at pilot-scale, as summarized in Figure 2.2, the level of developments from the published experiences decreases progressively from upstream to downstream to application. In general, the upstream bioprocess technology developments support that a PHA-rich biomass can be consistently produced and is adaptable within a wide range of different scenarios. Even though there are continued fundamental research questions yet to be answered, it is already technically feasible to generate mixed cultures highly enriched in PHA producing biomass, either through specialized enrichment processes (*enrichment accumulation* approach) or by means of mainstream biological wastewater treatment (*direct accumulation* approach). *Enrichment accumulation* and *direct accumulation* methods are complimentary to one and another, and as such offer a flexibility to exploit regional catchments of organic waste streams in a way that can maximize productivity while meeting other constraints and requirements depending on context and feedstock. The obtained functional biomass can be used to produce a range of co-polymer blends from a wide mix of possible simple and complex fermented VFA-rich industrial and municipal feedstocks. Independent of the feedstock used for the polymer accumulation bioprocess, PHA yields on substrate can be close to theoretical maximum levels, and significant PHA contents may be robustly achieved even though the maximum biomass PHA content before downstream processing may currently be considered to be the characteristic difference between *enrichment accumulation* and *direct accumulation* approaches. The difference of biomass PHA content might affect the choice of the DSP and the quality of the final extracted polymer. However, the basic outcomes and requirements for scaling-up of the process are equally valid for *enrichment* or *direct accumulation* PHA production methods. One can even expect that any practical differences between so-called *enrichment* and *direct* accumulation processes would become trivial if, for instance, the *direct accumulation* approach was applied with waste activated sludge that could produce biomass with up to 0.6 gPHA/gVSS. Even though the production potential of biomass can be further optimized especially for municipal activated sludge, one can conclude the pilot-scale experience in PHA-rich biomass production to date

positively motivate an initiative to scaling-up production of the PHA-rich biomass semi-product.



While production of PHA-containing biomass has been well studied even at pilot-scale, projects reporting on piloting experience with the downstream processes of PHA recovery are lacking. DSP could be done with both solvent-based and water-based methods. A solvent-based DSP typically includes process steps of dewatering, acidification, drying and solvent extraction (Werker et al., 2020). Heat for drying and non-chlorinated (solvent) extraction are principal recovery costs (Fernández-Dacosta et al., 2015). Drying costs are linked to the amount of moisture per total mass dried, and extraction costs are limited to the volume of mass that can be processed per batch. A high degree of solvent recovery and its reuse is furthermore important to the environmental and economic performance of solvent-based methods. Spent non-PHA biomass is a retained resource with application for its chemical and heat value. On the other hand, DSP by water-based methods involve steps of selective non-polymer biomass solubilization followed by granule separation and associated washing steps (Lorini et al., 2021b; Burniol-Figols et al., 2020; Kosseva and Rusbandi, 2018). It may be expected that water-based methods become more costly due to a greater amount of chemical consumption to remove non-polymer biomass the lower the biomass PHA content. The solubilized solids including digestion chemicals as well as the released solubilized biomass organic, nitrogen and phosphate contents generate a wastewater that must be treated. Consequently, solvent extraction methods might be more applicable at larger scales for processing PHA-rich biomass with moderate polymer content. Water-based methods may be initially more attractive at smaller scales and with PHA-rich biomass having higher biomass PHA content.

To our knowledge, there is only one detailed reported experience on pilot-scale DSP and production quality control for microbial community-based PHA (Werker et al., 2020). The polymers were recovered from dried biomass by using simple alcohols and/or acetone (Werker et al., 2020). Optimal conditions of recovery were influenced by the average co-polymer composition, molecular mass, particle size, and polymer-in-biomass chemical and thermal stability. The degree of polymer decomposition during recovery was predictable, and a pure polymer of

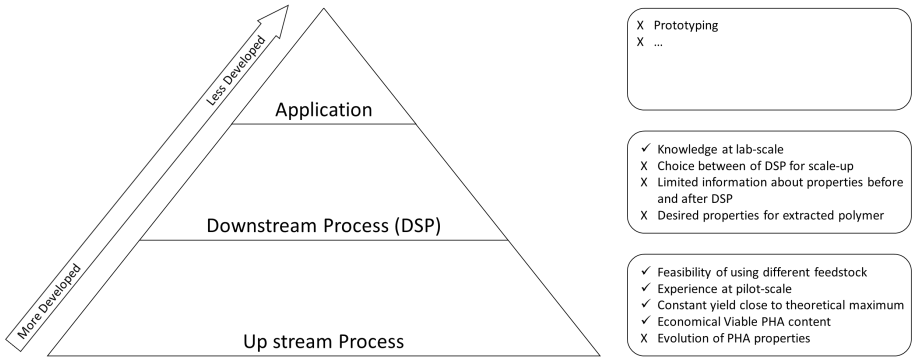


Figure 2.2. Summarized current development levels for PHA production process

commercial quality (98%) could be recovered even with biomass containing 0.4 gPHA/gVSS.

The challenge of developing a specific DSP is an uncertainty of what the feedstock quality, type of polymer, and requisite polymer properties will be, initially, in scaling-up efforts. Up until now the published research is normally linked to the goal to obtain a high purity polymer with moderate to high molecular mass. However, the question and demands of purity and molecular mass are very much linked to the polymer specifications for application in a product. In many cases, the base polymer properties are modulated in formulations that influence, for example, crystallization in processing. These formulations are best developed with supply of the same type of pending commercial grades of the polymer. Currently, the majority of the PHA production development is still on microbial PHA production supposing that a market exists because biopolymers are needed due to a crisis of plastics in the environment. However, no market can exist until a supply is available. The dilemma is without significant amounts of the commercial prototype for the polymer, one cannot test the feasibility of specific types and grades of the prototype PHAs for opportunities within promising and sometimes unforeseen applications. Therefore, the next steps for scaling-up microbial community-based PHA production are challenging because optimal downstream methods depend on the upstream (type of PHA, PHA-in-biomass quality, biomass quality, PHA content), the application intent (scale of production, polymer property quality window, reg-

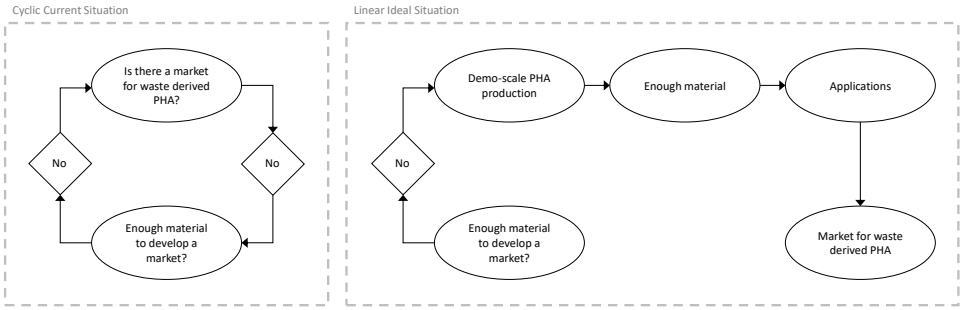


Figure 2.3. Depiction of dilemma in current scaling-up efforts of microbial community-based PHA production and how a demo-scale installation may contribute to support potential industrial implementation.

ulatory frameworks) and the overall value chain economic viability starting with the supply of VFAs.

To break this Catch-22, next steps of scaling-up upstream processes of production of PHA-rich biomass, such as a relevant demo-scale installation, can provide for a prototype stream of representative raw material, to more fully establish methods and process for the product recovery, as illustrated in Figure 2.3. Even the establishment of a demo-scale installation of PHA-rich biomass production will naturally involve a context with specific potential volumes in supplies of organic waste and/or wastewater conversions to PHA-rich biomass in the scope of methods from *direct accumulation* to *enrichment* culture bioprocesses. Scaling-up embodies a business understanding to reliably supply a mass of polymer for a given price towards commercially viable application(s). The type of fermented streams used for PHA production will also determine upstream engineering details of process volumes etc, as well as the specifics of the polymer type, i.e. co-polymer blend composition and this will, in turn, govern range and scope of application. Range and scope of application may furthermore determine the most appropriate methods for commercial DSP. Thus, the most relevant developments require a context of the specific organic materials being converted for the initial (commercial) scale of production, the regional growth potential in scale of organic material supply, and an application that fits with the potential market and specificities of the sup-

ply chain. The involvement and close collaboration of stakeholders all the way from feedstock, upstream process, DSP and application brings extra challenges. However, such cooperation is required to move the microbial community-based PHA production efficiently forward technically as well as economically.



## 2.4. Application case studies

In the scientific and popular literature, PHAs are purported to be drop in substitutes for traditional polymers for the plastics industry with properties similar to polypropylene and polystyrene. Biopolymers are implied to replace traditional plastics in many mainstream applications including durable products, as well as short term use applications, like packaging. This market for PHAs would require demanding DSP to produce PHAs of sufficient general quality for the open market. It would also require a scale of supply that would be unrealistic to expect to achieve, at least in the short term. The pilot studies to date give an impression of what the scale of supply may reasonably be.

As a practical example, if the OFMSW produced every year in the European Union (38,802 kt OFMSW; year 2018) was collected and *all* of it was used to produce PHAs, around 394 kt PHA could be produced per year (Appendix). The estimated amount of PHA that can be produced from this supply of organic waste represents only 0.6% of the current production scale and demand of petroleum-based polymers. The difference in production scale suggest that PHA will not be able to readily compete with traditional demands for polymers from petrochemical industries and highlights the importance of developing applications for PHAs that do not pretend to compete with petroleum-based polymers or mislead to unreasonably offer a drop in solution to global plastic pollution problems. Applications should be based on the unique PHA polymer properties, including but not limited to biodegradability. Even from the above very simple mass balance, it is clear that niche applications are required that can match realistically a scale in production volume that reliably secures stakeholder commercial investments in the supply chain. Towards this end, two application case studies are provided below to illustrate and develop the thoughts of strategy and challenges in next steps for scaling-up from published demonstrated pilot-scale technical successes to date at pilot-

scale.

Self-healing concrete is a type of concrete where the spores of specific haloalkaliphilic limestone-producing bacteria and poly-lactate polymer are mixed with the concrete raw materials in the production process (Jonkers et al., 2010). When cracks are formed, oxygen and moisture enter the concrete, this activates the spores. The growing bacteria consume the poly-lactate and generate limestone. The limestone seals the cracks preventing further crack growth and blocking further intrusion of water, prolonging the service life-time of the concrete. Due to the longer expected material service life-time, an environmental benefit comes also from lower associated concrete production $CO_2$ emissions (Wiktor and Jonkers, 2016). PHA could be used as an attractive substrate replacing lactate, as it is expected to be cheaper to use than lactate. In a recent publication, the feasibility of using waste-derived PHA for self-healing concrete has been demonstrated (Vermeer et al., 2021).

Controlled (or slow) release fertilizers (CRFs) are fertilizer products with embedded nutrients that are released to the soil in pace with plant growth requirements (Azeem et al., 2014). As a consequence, CRFs provide corollary benefits by mitigating nutrient run-off with associated environmental impacts of ground water contamination, eutrophication of surrounding water bodies, and emissions of greenhouse gases (nitrous oxide) (Boyandin et al., 2017). Commercially available CRFs apply a poorly biodegradable fossil-based polymer skin around a selected fertilizer pellet formulation as a diffusion membrane. The leftover of the polymer skin will remain as a micro-plastic waste in the soil. To exploit the benefits of CRFs without spreading micro-plastic waste and in accordance with the EU Fertilizing Products Regulation (Regulation (EU) 2019/1009), PHAs as bio-based and biodegradable polymers are an alternative to achieve the CRF function. There are several reports on the feasibility of different formulations and methods using high purity commercially available pure culture derived PHAs in CRFs (Boyandin et al., 2016; Volova et al., 2016; Boyandin et al., 2017).

Self-healing concrete and CRFs are emerging technologies which have a modest but significant market and raw material demands. As an example, 5000 t/year of PHA may be estimated to be required if *all* the applied mineral fertilizer in

Europe would be substituted by PHA-based CRFs (Appendix). At the same time, waste activated sludge from a 1 million p.e. municipal wastewater treatment plant could have the capacity to produce PHAs in the order of 2500 t/year of PHA Bengtsson et al. (2017b). This example illustrates context of a niche application where initial supply chains may readily target a valued market within the scope in scale of demand. The application developments would be best achieved when production capacity and market demand can be in balance. The demands from these emerging technologies will be modest to begin, but so will the supply chain too. One might think, for a single emerging technology, the PHA demand seems small. However, it is important to keep in mind that the expected market demand and supply chain infrastructure can be stimulated to blossom within an evolution of emerging applications. This evolution is driven from ongoing discovery from exploiting unique properties of PHAs produced from specific organic waste streams once there is a commercial scale supply.

These two emerging technologies, as examples, were selected to illustrate their common question of relevance in scaled-up supply for the polymers given a particular waste organic feedstock. They also contrast in quite different considerations of up- and downstream methods and process of production. These considerations enable the delivery of polymers with different qualities that are linked to very specific objectives in the applications. For self-healing concrete, it is anticipated that the molecular weight and the HV content of the PHA are of limited importance to the polymer function in the application. However, the concrete quality may be sensitive to other impurities which can potentially harm the strength of the concrete. Therefore, purity requirements for the polymer recovery are directed towards avoiding very specific kinds of biomass components rather than presence of trace contaminants of a polymer sold on the open market. For CRFs, the biodegradation rate of PHAs will affect the delivery of the fertilizer. The biodegradability of the PHAs are affected by the environmental conditions and the polymer properties (Emadian et al., 2017; Kale et al., 2007; Ferreira and Akesson, 2020). Crystallinity, HV content and thermal stability are essential factors for the biodegradation of the polymer (Zaheer and Kuddus, 2018; Ferreira and Akesson, 2020). Crystalinity affects the accessibility of the PHA degradation enzymes (Zaheer and Kuddus,

2018). The higher the crystallinity of the polymer the lower the degradation rate is expected to be. HV content will also affect the crystallinity of the polymer (Yu et al., 2005; Yu, 2009; Porter and Yu, 2011). A lower average HV content correlates to less crystalline polymers with higher melting temperature. Higher melting temperature also contributes to decreasing PHA biodegradation rates (Zaheer and Kuddus, 2018).

As discussed before, piloting experience has shown that these properties can be predicted and modulated during the PHA production process. Tuning the bioprocesses and DSP can become very technically and economically strategic when the principal objectives are linked to specific kinds of impurities (self-healing concrete) and/or crystallinity and Mw (CRFs). The DSP can furthermore be efficiently integrated into conversion steps for applications if the polymers do not need to be recovered and sold first as pure chemicals on the open market. The scaled up production and polymer quality is best to not be considered with respect to references of absolute quality targets, but with respect needs for a specific application.

## 2.5. Research and development directions

Based on the review of the published piloting research and developments, the following elements are recommended as necessary to bridge specific context for necessary linking between the upstream and downstream research and development efforts.

### 2.5.1. Upstream

- Understanding the evolution of polymer properties in the PHA accumulation process;
- Focus on factors underlying ambiguities in observed differences in the performance of selection in PHA enrichment reactors and municipal WWTPs;
- Development of consistent strategies to maximize PHA contents and yields in PHA production by municipal waste activated sludge;
- Development of strategies to mitigate flanking metabolic activity as integral

part of the PHA accumulation process with municipal activated sludge;

### 2.5.2. Downstream

- A focus on niche applications for microbial community-based PHA based on the unique polymer properties, including but not limited to biodegradability;
- The impact of different downstream processes on PHA product specifications needs to be better investigated for a product oriented DSP;

### 2.5.3. Full-scale context

- A realistic context of feedstock supply for commercial scale production at demo-scale that would provide for production of the representative type of PHA for the downstream processing and application engineering, as well as nurturing necessary stakeholder relations and commercial developments.
- Building sound business cases with help of substantive techno-economic evaluations using regional data from both public and private stakeholders, represented in the supply chain, towards understanding reliable flows in material supply that could support viable business(es) driven by niche applications with market potential suitable in scale to the emerging commercial supply;

The aim of this thesis was to study and optimize the direct use of municipal activated sludge for PHA production. Therefore, particular attention was given to those challenges related to the upstream and not downstream or full-scale context.

## 2.6. Conclusions

Microbial community-based PHA production from organic waste and wastewater has been shown at pilot scale to be a ready technology that offers meaningful contribution for resource recovery. Commercial quality polymers can be consistently produced. Main knowledge gaps remain in the bioprocess and downstream processing in linking relationship to the production methods for PHA applications

with specific product specifications. Further, commercial production will require a greater depth in fundamental understanding of the polymer characteristics towards process control of the polymer properties over the different stages in the whole PHA production chain from organic waste to value added products and services.

## Appendix

From CBS data (CBS (Centraal Bureau voor de Statistiek), 2020a,b), in 2018 in The Netherlands 1,487,000,000 kg OFMSW were produced (87 kg per person). Based on Moretto et al. (2020b), on average OFMSW has 0.132 kgVS/kgOFMSW and 13 kgVS are required to produce 1 kg PHA. This means that 15,098 tPHA (15 ktPHA) can be potentially produced from organic waste per year in The Netherlands. If the same calculation is done for Europe, assuming a population of 446 million, 393,990 tPHA/year (394 ktPHA) could be produced. These numbers can be compared with the traditional plastic industry. Europe produced in 2018, 62 mt of plastics (62,000 kt) (PlasticsEurope, 2019), which means that the PHA production would represent only 0.6% of the plastic industry at European level. These numbers strongly suggest that PHAs will not be able to compete with traditional petroleum-based polymers and should find other entry markets.

About 10 million tons of mineral fertilizer are applied annually in Europe (European Commision, 2020). At the limit of ideal nutrient delivery, CRFs would reduce fertilizer demand by nominally 50% (Mosier et al., 2013). Then, if all the fertilizer application in Europe was to be based on CRFs, and the mass of PHA in the fertilizer was 0.1% of the total weight, the supply to meet EU market demand for PHAs based CRFs would be in order of 5000 t/year of PHA.

*To Chapter 5*

# 3

# Process conditions affect properties and outcomes of PHA accumulation in municipal activated sludge

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## Abstract

*The developments of mixed culture polyhydroxyalkanoate production has been directed to maximize the biomass PHA content with limited attention to polymer quality. Direct comparison of PHA accumulation literature is challenging, and even regularly contradicting in reported results, due to underlying differences that are not well expressed. A study was undertaken to systematically compare the commonly reported process conditions for PHA accumulation by full-scale municipal activated sludge. A biomass acclimation step combined with a pulse-wise feeding strategy resulted in maximum average PHA contents and product yields. pH control and active nitrification did not result in observable effects on the PHA productivity. Under these conditions a high molecular weight polymer (1536 ± 221 kDa) can be produced. Polymer extraction recoveries were influenced by the PHA molecular weight. A standard protocol for an activated sludge PHA accumulation test including downstream processing and standardized extraction has been developed and is available as supplementary material.*

## 3.1. Introduction



Waste activated sludge is produced as a by-product in biological wastewater treatment plants (WWTP). Its production, and subsequent disposal, contributes significantly to WWTP operational costs (Appels et al., 2008). Within regionally based circular economies, waste activated sludge does not necessarily need to be a waste (Tyagi and Lo, 2013). Municipal waste activated sludge can instead be a resource for the production of alginate-like exopolysaccharide or polyhydroxyalkanoates (PHAs) (Kehrein et al., 2020). It can be a raw material input for a PHA production process if volatile fatty acid (VFA) rich streams can be made sufficiently available as feedstock (Bengtsson et al., 2017b).

PHAs are intracellularly stored bacterial energy and carbon reserves that can be extracted from biomass and applied in the polymer industry (Philip et al., 2007). PHAs represent an alternative to non-biodegradable oil-based polymers, but the current levels of industrial production are not able to compete economically with the traditional oil-based polymer industry (Estévez-Alonso et al., 2021a). PHA production methods are evolving to meet ongoing goals of increased production capacity with improved economy. Waste activated sludge from a biological WWTP and fermented organic waste and wastewater streams can be used for PHA production. Waste activated sludge can be used directly for PHA production or used as inoculum for the enrichment of a PHA-storing biomass (Estévez-Alonso et al., 2021a). The *enrichment* strategy has been investigated over two decades (Estévez-Alonso et al., 2021a; Valentino et al., 2017). However, a *direct* accumulation approach has received less research attention (Arcos-Hernandez et al., 2013).

Considerable efforts at lab and pilot scales have been directed to establish optimal operational conditions that maximize the biomass PHA content. Examples of the operational conditions that have been studied until now are: the modulation of nutrient concentrations in the feedstock (Silva et al., 2017; Valentino et al., 2015; Johnson et al., 2010a; Punrattanasin et al., 2006; Wen et al., 2010; Chinwetkitvanich et al., 2004; Cavaillé et al., 2013), redox conditions (Liu et al., 2011; Pratt et al., 2012; Punrattanasin et al., 2006; Moralejo-Gárate et al., 2013), the feeding composition and feeding strategy (Serafim et al., 2004; Palmeiro-Sánchez et al., 2016b; Albuquerque et al., 2011; Valentino et al., 2015; Chen et al., 2013; Ma et al.,

2000; Chen et al., 2015), temperature (De Grazia et al., 2017; Johnson et al., 2010b; Chinwetkitvanich et al., 2004), pH control and salinity (Palmeiro-Sánchez et al., 2016b; Mulders et al., 2020a; Chen et al., 2013), presence or absence of nitrifying bacteria (Fra-Vázquez et al., 2019), and different biomass pre-treatments (Morgan-Sagastume et al., 2019). Direct comparison between these studies is challenging due to the differences in the selected PHA-storing biomass and applied process conditions for PHA accumulation. For instance, studies on the influence of nutrients on the accumulation of PHA have shown contradictory results. Johnson et al. (2010a) reported that maximum expressed cellular PHA content required the absence of nutrients in the feedstock. To the contrary, Valentino et al. (2015) found higher expressed PHA content when nutrient limiting conditions in the feedstock were applied. In these two studies, the PHA-storing biomass was enriched at different temperature, solid retention times and cycle length and furthermore, in the PHA accumulation reactor the feeding strategy, temperature and pH range were also different. This example illustrates contradicting outcomes and suggests that underlying context is an important factor. Most of these studies have also used specifically enriched biomass for the PHA accumulation, in part, to assess for the influence of the selection pressure on the biomass production and quality. Systematic evaluations for PHA accumulation with waste activated sludge, without further enrichment, are lacking in the research literature.

Optimal outcomes for industrial PHA production are a maximal quantity with consistent quality. Mixed culture PHA production typically produce co-polymer blends of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or PHBV. Most of the studies mentioned above have focused on the maximization of the biomass PHA content without evaluation of quality other than the average co-polymer composition (Silva et al., 2017; Palmeiro-Sánchez et al., 2016b; Albuquerque et al., 2011). Few studies have explored how accumulation conditions can influence the polymer quality and/or consistency of quality (Lorini et al., 2021a; Werker et al., 2020; Laycock et al., 2013). Polymer quality relates to the polymer mechanical and thermal properties. Polymer mechanical and thermal properties are affected by the co-polymer composition and the polymer molecular weight. The co-polymer composition is mainly determined by volatile fatty acids composition in the feedstock,

that would result in the production of the monomer 3-hydroxyvalerate (3HV) or 3-hydroxybutyrate (3HB) (Werker et al., 2020). Polymer thermal properties are directly related to the average 3-hydroxyvalerate content assuming a random monomer distribution in the blend. However, control of the polymer molecular weight outcomes are poorly understood with few publications on the subject (Serafim et al., 2008; Bengtsson et al., 2010; Albuquerque et al., 2011; Palmeiro-Sánchez et al., 2016b; Bengtsson et al., 2017b; Lorini et al., 2021b; Werker et al., 2020; Lorini et al., 2021a; Rodrigues et al., 2022).



The aim of this work was to compare selected commonly reported process conditions for PHA accumulation using full-scale municipal waste activated sludge as the PHA-producing biomass. Principal factors identified from the research literature were biomass acclimation, feeding strategy, pH control and, presence of nitrifying bacteria. Polyhydroxybutyrate was produced with acetic acid to enable a study focused on how accumulation conditions influenced outcomes of polymer quantity and molecular weight quality. As a result, a standard protocol for an activated sludge PHA accumulation test including downstream processing and standardized extraction has been developed and it is available as supplementary material.

## 3.2. Materials and methods

### 3.2.1. Experimental set-up

PHA accumulation tests were performed over 24 h in a 1 L double-jacketed glass bioreactor at 25 ± 0.1°C. In cases with pH control (Table 3.1), pH was maintained at 7.5 ± 0.1 with feedback control dosage of 1 M HCl. The airflow rate was fixed at 1 L/min (MV-302, Bronkhorst, Germany) and agitation at 150 rpm was by standard three-bladed turbine (R60, CAT Scientific, Germany). Dissolved oxygen and pH probes (COS81D and CPS11D, Endress & Hausser, The Netherlands) were coupled to a 4-channel transmitter (Liquiline CM444, Endress & Hausser, The Netherlands) and measurements were logged every 10 s. Probes were calibrated according to manufacturer instructions for each assay. Substrate feed dosing diaphragm pumps (Stepdos 10, KNF, The Netherlands) were actuated by PLC (Logo!

8 and Logo! TDE, Siemens, Germany).

### 3.2.2. Sludge source and feedstock

Waste activated sludge from the municipal WWTP Bath (Rilland-Bath, The Netherlands) was used for PHA accumulation. Fresh gravity belt thickened waste activated sludge (56 ± 4 gTS/kg and 39 ± 3 gVS/kg) was delivered batch wise by courier every two weeks in 20 L carboys and stored at 4°C pending experiments. In total, 14 distinct batches delivered over a period of six months were used for these experiments.



The accumulation feedstock, with nutrients ratio 100:1:0.05 (COD:N:P by weight), was prepared with tap water as follows: 50 g/L acetic acid, 1.91 g/L $NH_4Cl$, 109.6 mg/L $KH_2PO_4$. The feedstock pH was adjusted to 5.5-6 with KOH pellets.

### 3.2.3. PHA accumulations with different process conditions

A total of 25 PHA accumulation experiments were performed to evaluate an influence of process conditions (Table 3.1). For each experiment, an aliquot of the stored activated sludge was diluted with tap water to nominally 2.5 gVSS/L. The biomass was conditioned at 25 ± 0.1°C by aeration overnight to establish a baseline of endogenous microbial activity. Allylthiourea (20-50 mg/L) was then added

Table 3.1. Overview of the accumulation tests and the operational conditions used. F/M ratio = initial food to microorganism ratio; ATU = allylthiourea.

| Test | Feeding | Pulse F/M ratio | pH control | ATU | Acclimation | N° of tests |
|---|---|---|---|---|---|---|
| - | - | $mgCOD_{HAc}/gVSS$ | - | - | - | - |
| 1 | Pulse-wise | 20 | Yes | Yes | No | 3 |
| 2 | Pulse-wise | 60 | Yes | Yes | No | 4 |
| 3 | Pulse-wise | 60 | Yes | No | No | 3 |
| 4 | Pulse-wise | 60 | No | No | No | 4 |
| 5 | Pulse-wise | 60 | No | No | Yes | 5 |
| 6 | Pulse-wise | 400 | Yes | Yes | No | 3 |
| 7 | Continuous | 200 | Yes | Yes | No | 3 |

directly to the reactor in some cases (Table 3.1). The air supply was interrupted for 30 min to assess the endogenous respiration level. The subsequent re-aeration period was used to determine the oxygen mass transfer coefficient $k_La$. PHA accumulation was then started according to pre-selected protocols of feeding. The feedstock solution was dosed continuously or in fixed-volume pulses to target a selected maximum peak substrate concentration of 50, 150 or 1000 $mgCOD_{HAc}/L$. These targeted substrate concentrations were chosen to reach an initial food to microorganism ratio (F/M ratio) of 20, 60 and 400 $mgCOD_{HAc}/gVSS$, respectively. Continuous excess feeding was started with a pulse of 500 $mgCOD_{HAc}/L$ and with subsequent continuous substrate addition. The rate for substrate addition in the continuous excess feeding was based on the average rate of substrate consumption from previous experiments. The continuous excess feeding aimed to reach a food to microorganism ratio of 200 $mgCOD_{HAc}/gVSS$. Fixed-volume pulse inputs were controlled from on-line monitoring of dissolved oxygen concentrations, as previously reported (Valentino et al., 2015; Werker et al., 2013a). PHA accumulation tests with fixed-volume pulses of 150 $mgCOD_{HAc}/L$ were also used to investigate for an influence of pH control at 7.5, presence of active nitrifying bacteria, and short-term biomass acclimation. For acclimation, the biomass was subjected to three feast and famine cycles before the start of the accumulation process (Morgan-Sagastume et al., 2019). Feast conditions were generated with a pulse input to reach a maximum substrate level of 150 $mgCOD_{HAc}/L$ and the duration of the feast phase was monitored by dissolved oxygen concentration. The applied famine period was three times longer than the feast time. After the third famine period, the accumulation feed-on-demand process was started automatically.



### 3.2.4. Biomass downstream processing and polymer extraction

At the end of the experiment, the reactor mixed liquor was collected and acidified to pH 2 with 95% $H_2SO_4$. Suspended solids were recovered after centrifugation (10000 rcf and 4°C for 20 min). Subsequently, the recovered wet PHA-rich solids were heated at first for 30 min at 120°C followed by overnight drying at 105°C. PHA quantity and quality in the dried biomass were assessed by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and pyrolysis

with gas chromatography combined with mass spectroscopy (PyGCMS) (see analytical methods).

The polymer was extracted in 13 mL dimethyl carbonate (DMC, Sigma-Aldrich ReagentPlus®, 99%) from weighed ground biomass samples to target a maximum theoretical polymer concentration of 20 mgPHA/mL. Ground samples were first weighed and re-dried for 30 min at 105°C in tare weighed 20 mm diameter) glass digestion tubes (Hach, LZP065). A weighed mass of DMC was added, and tubes were sealed with respective tare weighed caps. Tube contents were vortex mixed and placed in a 140°C pre-warmed heater block (Hach-Lange, LT200) for 20-minute extraction time wherein contents were vortex mixed briefly every 5 minutes. After the extraction time, and the final vortex mixing, tubes were transferred to an 80°C pre-warmed heater block (Grant, QBD4) and biomass was settled by gravity. About 11 mL of the solvent solution was carefully decanted to pre-warmed (90°C) 15 mL Falcon tubes excluding most suspended solids. The warm solution was centrifuged (up to 9418 x g over 2 minutes), with pre-heated (90°C) tube inserts to remove remaining suspended solid fines from the polymer solvent solution. A weighed amount (about 10 mL) of the still hot solution was carefully decanted directly into a tare weighed soda-lime glass petri dish (Duran, 90 mm diameter) that was placed on a level drying scale (Satorius MA37). Solvent was evaporated at 85°C with gentle heating and formed a dried solution cast polymer film (< 0.1% mass change over 1 minute). Added and removed masses of solids and solvent (± 1 mg) were followed at each step of the extraction protocol for making mass balances. The extracted mass was derived from the solution concentration, estimated from film casting, and the known total mass of extraction solvent used. Extraction yields were calculated relative to the amount biomass in the extraction tube after the initial re-drying before solvent addition. The recovered polymer thermal properties were characterized by TGA and DSC, and weight average molecular mass was estimated by solution rheology (see analytical methods).

### 3.2.5. Analytical methods

The PHA accumulation process was monitored by logging DO, pH and temperature. Three 15 mL grab samples were taken at selected times. Suspended solids

were separated from the 15 mL of the mixed liquor by centrifugation (3250 rcf and 4°C for 20 minutes). The supernatant after membrane filtration (0.45 $\mu$m pore size filters) was stored at -20°C pending liquid analyses. The harvested biomass pellet dry weight and ash contents were measured according to Standard Methods (105°C drying and 550°C ashing) and referenced to the sample volume for estimated total and volatile suspended solids (TSS and VSS), respectively. Soluble chemical oxygen demand (sCOD) was determined by Hach-Lange (Germany) LCK014 and LCK314 test kits. Acetic acid concentration was determined by ultra-high pressure liquid chromatography and ammonium, nitrite, nitrate and phosphate concentrations were determined by ion chromatography, as previously reported (Estévez-Alonso et al., 2021b). 15 mL of mixed liquor were directly acidified to pH 2 with 95% $H_2SO_4$. After thorough mixing (5 min) suspended solids were collected (3250 rcf and 4°C for 20 min). The biomass pellet was dried at 105°C. Dried pellets were ground by hand with mortar and pestle for polymer characterization before and after extraction.

Thermogravimetric analysis (TGA2, Mettler-Toledo), for PHA content with respect to total solids (TS) and volatile solids (VS) as gPHA/gTS and gPHA/gVS, and for thermal decomposition temperature ($T_d$), was performed as previously described (Chan et al., 2017). Representative sub-samples of about 5 and 2 mg of dried biomass or recovered polymer were used, respectively. The method includes estimation of sample residual moisture and PHA contents as well as the overall organic and inorganic fractions. Briefly, pre-weighed samples were inserted to the TGA at 80°C with nitrogen purge gas at 50 mL/min. Temperature was increased (10°C/min) to 105°C and held for 15 minutes wherein moisture (or residual solvent) content could be estimated. Temperature was increased (10°C/min) to 550°C and held for 30 minutes. PHA mass could be estimated from the characteristic rapid mass loss occurring between 225 and 350°C. At 550°C the purge gas was changed to air at 50 mL/min. Sample ash content was estimated by weight loss at 550°C with air atmosphere. Reference samples included a PHA-rich biomass with known PHA content (45.1 ± 0.6 gPHA/gVS), and pure PHB (> 98% purity, Biomer, Germany). Temperature was calibrated based on Curie temperature with a nickel standard following Mettler-Toledo methods.

Differential scanning calorimetry (DSC 3+, Mettler-Toledo) was also performed based on previously described methods (Chan et al., 2017) and with nominally 5 and 2 mg dried biomass and recovered polymer samples, respectively. Sample PHA purity was estimated from TGA measurements. Weighed samples were inserted and held for 5 minutes at -70°C with nitrogen purge gas at 50 mL/min. A first heat and quench cycle followed with heating and cooling at 10°C/min to 185°C and back to -70°C. A second heat ramp at 10°C/min to 185°C was applied followed by quenching (-100°C/min) to -70°C after 0.5-minute hold at 185°C. A third heat ramp at 10 °C/min to 185°C was applied followed by quenching (-30°C/min) to -70°C, after 0.5-minute hold at 185°C. Finally, the sample was heated at 10°C/min to 40°C. Melt and crystallization enthalpies were estimated with respect to the mass of PHA in the sample. Reference samples included pure PHB (>98% purity, Biomer, Germany) and an in-house PHBV standard (34% wt. HV content). The instrument was calibrated with pure zinc and indium standards according to Mettler-Toledo methods.

Polymer weight average molecular mass (in kDa) was estimated by solution rheology and intrinsic viscosity $[\eta]$ measurements (dL/g). Polymer solutions (about 10 mL) were generated by combining weighed amounts of polymer dried film and DMC to result in known concentrations in the order of 15 mgPHA/mL. Polymer concentration was corrected for the film PHA purity that was estimated from the TGA measurements. Solutions were heated to dissolve the polymer in sealed glass digestion tubes (Hach, LZP065) for 5 minutes at 140°C, vortex mixed, then cooled and maintained in the heater block at 70°C. Viscosity of 5 mL aliquots of the heated solutions were measured at 60°C with a concentric cylinder measurement system rotating at a 75 $s^{-1}$ shear rate (Anton-Paar MCR102 with a CC17 standard measuring system). Viscosity was estimated every 10 seconds from the average torque and over 5 minutes for each sample. Relative viscosities were estimated with respect to measurements of the solvent without added polymer and the intrinsic viscosity was calculated with the Solomon-Ciuta equation (Elias, 2008):

$$[\eta] = \frac{\sqrt{2\left(\eta_{sp} - ln\left(\eta_r\right)\right)}}{c} \tag{3.1}$$

where $c$ is the PHA concentration (g/dL) in DMC, $\eta_{sp}$ is the specific viscosity, and $\eta_r$ is the relative viscosity. Validity of linearity assumptions for intrinsic viscosity estimated with this equation have been confirmed. Conversion of intrinsic viscosity to an estimated weight average molecular mass was made with the Mark-Houwink equation (Marchessault et al., 1970):

$$[\eta] = K\mathrm{M}_{\mathrm{w}}^{\alpha} \tag{3.2}$$



where K and $\alpha$ are empirical constants relating $[\eta]$ (dL/g) to molecular weight (kDa). These Mark-Houwink constants, logK (-2.016 ± 0.025) and $\alpha$ (0.7384 ± 0.0098), were calibrated in separate research developments with respect to parallel samples dissolved in chloroform and molecular weight determined by gel permeation chromatography (GPC) with GPC calibration to polystyrene standards similarly to previously reported (Chan et al., 2017).

Polymer monomer composition was assessed by pyrolysis with gas chromatography and mass spectroscopy (PyGCMS). The evaluation gave the weight percent of 3-hydroxyvalerate (3HV) in the accumulated poly(3-hydroxybutyrate-co-3hydroxyvaleraye) (PHBV). A Gerstel (Mülheim an der Ruhr, Germany) pyrolysis unit was used with a Thermal Desorption Unit (TDU) and a liquid nitrogen Cooled Injection System (CIS). Grab samples of about 50 µg polymer particles, or polymer containing biomass particles, were deposited into an open ended clean 25 mm long (Gerstel 018131) quartz tube with a small retaining plug of quartz wool. Samples were loaded for pyrolysis by a Gerstel MPS 2XL autosampler. Pyrolysis was carried out under helium gas flow (60 mL/min) starting at 50°C for 0.5 minutes followed by heating at 120°C/min up to 710°C for pyrolysis unit, and 350°C for the TDU. The complete pyrolysis/TDU cycle lasted 8 minutes with maximum pyrolysis and TDU temperatures maintained. Pyrolysis products carried by the gas flow were trapped in the CIS at -100°C on a quartz wool filled liner in solvent vent mode. Gas chromatography (GC, Agilent Technologies 6890N, Santa Clara, CA, USA) was employed using a Phenomenex ZB-5MS column (30 m, 0.25 mm diameter, 1 µm film thickness). The CIS and GC temperature programs were run in parallel enabling transfer and focus of analytes onto the head of the column with a split flow of 20 giving 1 mL/min constant helium flow in the column. CIS temper-

ature program was from -100°C for 0.02 min, and then up to 280°C at 600°C/min. The GC oven temperature program was from 70°C for 5 min, and then up to 290°C at 10°C/min. The detector after GC was an Agilent Technologies 5975 XL Mass Selective Detector (MSD). MS data was acquired in scan mode with mass-to-charge (m/z) ratios ranging from 15 to 550 in a total scan time of 0.649 seconds. Pyrolysis, TDU and CIS were driven with Gerstel Maestro Controller (version 1.4.21.1) and MPS (version 1.4.15.1) drivers. GC and MSD were controlled by Evo3/Enhanced Masshunter GC/MS Acquisition (version B.07.06.2704). Acquired TIC (total ion chromatogram) data was processed and integrated using MassHunter Quantitative Analysis (Agilent Technologies, version B.07.00) while monitoring for the specific ion chromatogram (SIC) m/z 86 and 100. The SIC gave qualifying ions as markers for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate, respectively, since the pyrolysis process converts 3HB to 2-butenoic acid and 3HV to 2-pentenoic acid through a dehydration reaction (Khang et al., 2021). NIST MS Search (version 2.0, 2008) was used to confirm the identity of 2-butenoic acid and 2-pentenoic acid; match percentage exceeded 85% for both compounds. The integrated TIC peak ratio for 2-pentenoic acid with respect to the sum of 2-butenoic and 2-pentenoic acids was found to correlate directly to the 3HV wt% in PHBV with standards (Aldrich PHBV standards with 0, 5, 8 and 12% wt. 3HV content) and extracted PHBV using DMC. Correlation between SIC and TIC integrated peaks from pure standards was used to estimate the 3HV wt% for PHBV pyrolyzed in the more complex matrix of PHA-rich biomass samples. The SIC signal allowed to avoid interference from overlapping signal coming from non-PHA biomass.

### 3.2.6. Data analysis

The data collected during PHA accumulation tests were corrected for effects of sampling, acid addition for pH control and addition of feedstock (Johnson et al., 2009b). The PHA content was expressed as mass fraction of the volatile suspended solids (gPHA/gVSS). The active biomass fraction or non-polymer biomass ($X_a$) was estimated as the total VSS minus PHA mass fraction. Active biomass was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (Johnson et al., 2009b). The trend of change in PHA content

was fitted by least squares regression to the empirical function:

$$\text{Biomass PHA content} = A_0 + A_1\left(1 - e^{-t/\tau}\right) \tag{3.3}$$

where $A_0$, $A_1$ and $\tau$ are constants that allow for estimation of rates as function of time (Bengtsson et al., 2017b). Initial and average specific production/consumption rates and PHA yields on substrate were estimated for time 2 and 24 h, respectively. The average PHA yields on substrate were calculated on a COD-basis by dividing the amount of PHA (given 1.67 gCOD/gPHB) produced by the amount of substrate (given 1.07 gCOD/gHAc) added. Average specific production and consumption rates were calculated based on the cumulative amounts of acetic acid, PHA, biomass and oxygen consumed, with respect to estimated active biomass levels (gCOD/g$X_a$/h).



## 3.3. Results and discussion

Direct comparison between mixed culture PHA accumulation experiences is challenging, and even contradicting in outcomes that have been reported, due to underlying differences in approach, context and operations. In the present study, a consistent approach, context and method of operations were applied. The undertaking started with comparison in outcomes of polymer accumulation quantity between the most commonly reported process conditions for PHA accumulation using full-scale municipal waste activated sludge as the PHA-storing biomass. First, continuous and pulse-wise feeding were evaluated. Pulse-wise feeding resulted in higher PHA contents and yields on substrate. Consequently, pulse-wise feeding was used as the platform for comparisons in further testing. Further testing included comparing process operating conditions such as pH control, the presence of active nitrification, and an influence of a biomass acclimation step prior to PHA accumulation. pH control and the presence of active nitrification were chosen because they can negatively affect the process performance due to an increase of salinity levels due to pH control and the reduction of available dissolved oxygen, due to the presence of active nitrification. Potential for influence of operations on the resultant polymer quality was characterized by solution

rheology, thermogravimetric analysis, differential scanning calorimetry and pyrolysis–gas chromatography–mass spectrometry.

### 3.3.1. PHA production by municipal activated sludge

Municipal waste activated sludge can be reliably used as the source of biomass for the production of PHAs. In the present work, PHA accumulation tests were performed with activated sludge samples collected over a period of six months. An example of a PHA accumulation test with pulse-wise acetate feeding (F/M = 60 $mgCOD_{HAc}$/gVSS) is given in Figure 3.1. Even though waste activated sludge was collected and used for PHA production at different time points, PHA contents and yields were observed to be consistently dependent on the process conditions used, as illustrated in Figure 3.2. This experience replicates previous pilot scale outcomes. The same municipal activated sludge, as was used in this work, consistently produced commercial quality grades of PHA in 52 production batches spread over more than 10 months (Bengtsson et al., 2017b). In the previous pilot study case, process conditions for PHA production were not changed during the experimental time and an average biomass PHA content of 0.41 gPHA/gVSS was obtained. Similar biomass PHA contents, 0.40 ± 0.01 gPHA/gVSS, were obtained

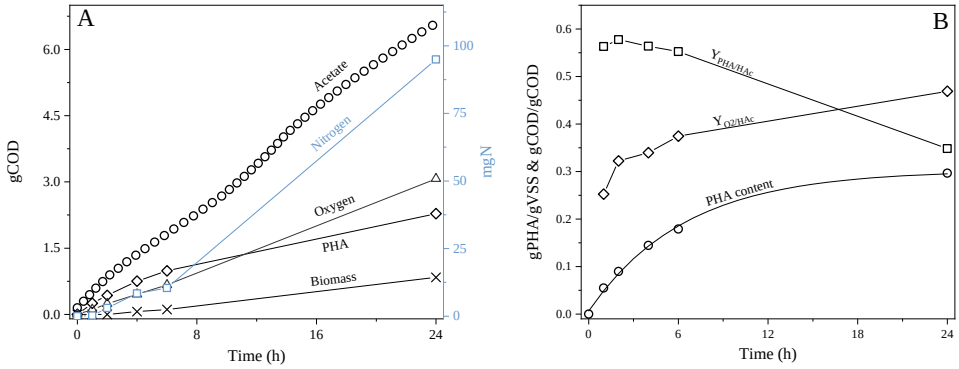


Figure 3.1. Example of a PHA accumulation test with pulse-wise feeding with pulses reaching initial F/M ratios of 60 $mgCOD_{HAc}$/gVSS. A) Acetate, ammonium and oxygen are shown as cumulatively consumed while PHA and biomass are cumulatively produced. B) Yields on substrate are the average yields on substrate at selected times. Symbols are measured values and lines are included to help readability.

in the present work under similar process conditions. These PHA contents are in line with expectations for PHA production with municipal activated sludge and are above the minimum PHA content required to make the process economically viable (Bengtsson et al., 2017b).

Process conditions are known to influence PHA storage and biomass growth (Serafim et al., 2004). In all tests performed in this work, a preference for PHA storage over biomass growth was observed. In the first hours of the accumulation, ammonium accumulated in the reactor and biomass growth was not measurable, as illustrated in Figure 3.1. By the end of the accumulation, low or negligible levels of ammonium, nitrite or nitrate were found in the reactor mixed liquor. Similarly, biomass respiration rates decreased in the first hours of the accumulation, but subsequently increased after 6-8 hours with maximum respiration rates towards the end of the accumulation. An increase in biomass growth was observed after 6-8 hours, as indicated by ammonium and oxygen consumption rates. This onset of active biomass growth can be explained by an adaptation of the biomass. It is likely that bacteria coming from conditions of famine would be in a state where the enzyme machinery necessary for cell growth would not be sufficiently induced, at least at the beginning of the accumulation test (van Aalst-van Leeuwen et al., 1997; Krishna and Van Loosdrecht, 1999). PHA production acts as a buffer for the excess substrate that cannot be directed for biomass growth. In the present work, substrate uptake rates reached the maximum value directly in the first pulse, but growth rates only slowly increased. Consequently, more of the consumed acetate was channeled towards PHA storage at the beginning of the accumulation test. Gradually more acetate was directed to metabolism for biomass growth. This shift in metabolism is supported by observations of decrease in PHA yield on substrate, increase in ammonium removal, and increase in organic solids during the accumulation experiment (Figure 3.1). A similar mechanism has been applied to explain the increase in growth rates observed after a pulse of substrate was given to an acetate limited chemostat culture (van Aalst-van Leeuwen et al., 1997). Research efforts should be directed to limit the growth response or on the contrary, to promote the growth of only the PHA-storing biomass fraction.

### 3.3.2. PHA accumulation performance under different feeding strategies

PHA contents and yields are consistently related to the applied feeding strategy. In general, higher PHA contents and average yields were obtained when the feedstock was supplied in pulses and not continuously (in excess). The feeding strategy with the lowest initial F/M ratio per pulse (20 $mgCOD_{HAc}/gVSS$) resulted in maximum PHA contents and average PHA yields on substrate, 0.34-0.35 gPHA/gVSS and 0.34-0.36 $gCOD_{PHA}/gCOD_{HAc}$, respectively. An overview of the observed PHA contents and yields on substrate can be found in Figure 3.2 and 3.3. Under these low F/M pulse feeding, higher initial polymer yields on substrate were obtained and ranged from 0.53 to 0.57 $gCOD_{PHA}/gCOD_{HAc}$. Feeding in pulses with low COD concentration resulted in a high number of pulses, of up to 142 pulses given within 24 hours. The effect of feedstock pulse-wise addition has been previously studied (Serafim et al., 2004, 2008; Albuquerque et al., 2011; Chen et al., 2013; Valentino et al., 2015). For instance, Serafim et al. (2004) found higher PHA contents when the feedstock was added in three pulses instead of one. On the contrary, Albuquerque et al. (2011) and Chen et al. (2013) found higher PHA contents when the feedstock was fed continuously compared to three or six pulses. Even though these

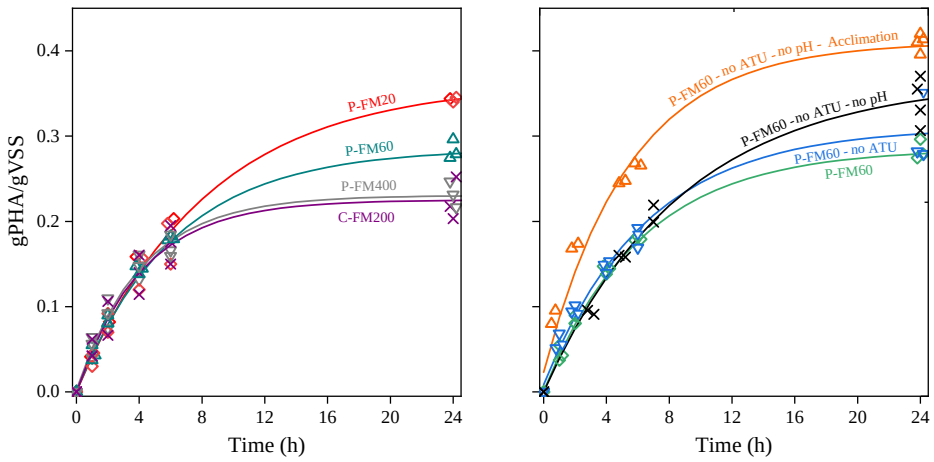


Figure 3.2. Overview of the PHA contents obtained in all the PHA accumulation tests with continuous excess feeding (C), pulse-wise feeding (P), uncontrolled pH, active nitrification and an acclimation step.

studies compared pulse-wise to continuous excess feeding, the number of pulses was small (three to six pulses) compared to the pulse-feeding tested in the present work with up to 142 pulses.

As expected, the feeding strategy also affected the biomass production rates. Higher biomass production rates were observed in tests where the feedstock was dosed continuously or in pulses reaching 400 $mgCOD_{HAc}/gVSS$ (Figure 3.3). When the substrate was added continuously or in pulses reaching 400 $mgCOD_{HAc}/gVSS$, the biomass production rate was 1.40-1.46 $gX/gVSS_0$, compared to those tests where the feedstock was dosed in pulses reaching peaks of 20 or 60 $mgCOD_{HAc}/gVSS$, where growth rates of 1.20-1.28 $gX/gVSS_0$ were observed. Even if the amount of biomass produced was higher, the average biomass yields on substrate were very similar between all the tested conditions. These trends were also observed in the ammonium removal rates, which were higher from the start of the test with continuous excess feeding or in pulses reaching 400 $mgCOD_{HAc}/gVSS$ (Figure 3.5).

Differences in PHA contents, yields and biomass production under different initial F/M ratios have been previously explained in the context of substrate inhi-

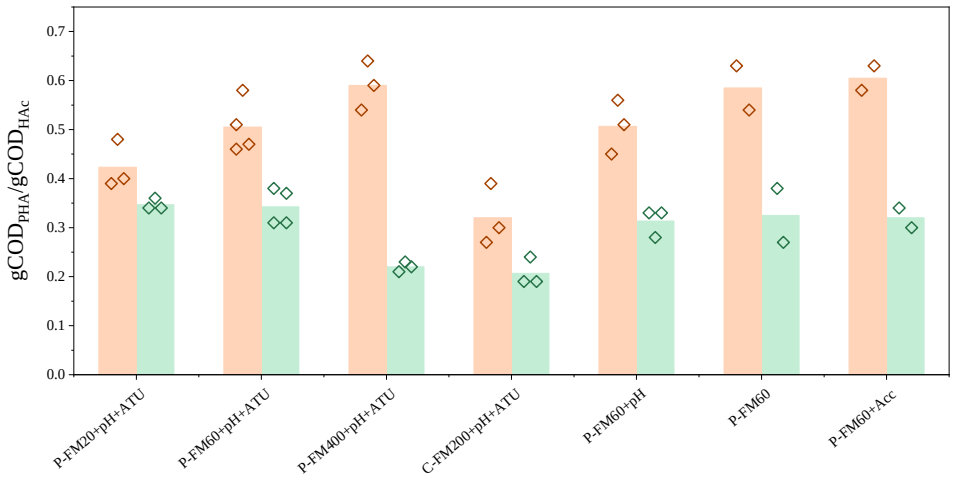


Figure 3.3. Initial (orange) and average overall PHA yields on substrate (green) with continuous excess feeding (C) as a function of initial F/M ratio, given pulse-wise feeding (P), pH control (+pH), nitrification inhibition (+ATU), and acclimation (+Acc).

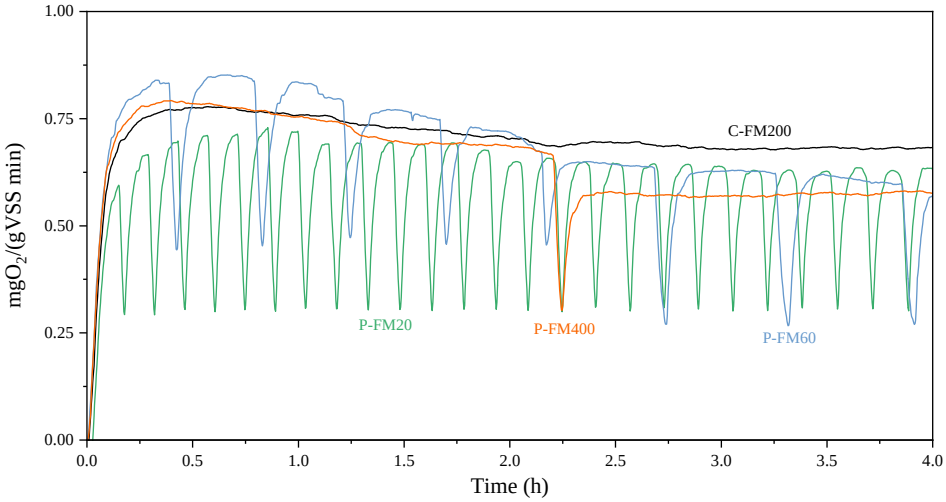


Figure 3.4. Specific oxygen uptake rate in PHA accumulation tests with pulse-wise (P) and continuous excess feeding (C).

bition (Serafim et al., 2004). If this is the case, acetate and oxygen biomass uptake rates should be lower at higher substrate concentrations. In the present work, initial respiration rates were very similar among experiments and in the range 0.6-0.8 $mgO_2/(gVSS\ min)$, as observed in Figure 3.4. No correlation could be found between a lower concentration of acetic acid per pulse and a higher initial respiration rate. For instance, in some tests with pulses reaching 400 $mgCOD_{HAc}/gVSS$, higher respiration rates were obtained compared to tests with pulses reaching 20 $mgCOD_{HAc}/gVSS$ and vice versa. Overall, oxygen consumption yields on substrate did not differ between tests with different feeding strategies, and they were in the range of 0.35-0.51 $gO_2/gCOD_{HAc}$.

The impact of dosing regime on the performance of accumulation requires careful consideration. The pulse-wise feeding strategy aims to still maintain the kinetics of polymer accumulation even though exogenous substrate supply begins to become exhausted before each subsequent pulse is triggered by a relative drop in respiration rate. A higher number of pulses will result in a higher number of interruptions in the availability exogenous of substrate. For instance, in the first six hours of a test with pulses reaching initial F/M ratios of 20 $mgCOD_{HAc}/gVSS$, 35 pulses were given. Between pulses, exogenous substrate was not available for

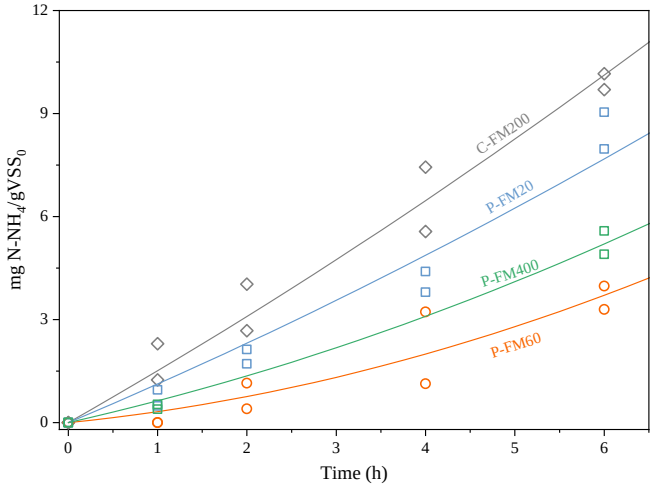


Figure 3.5. Differences observed in nitrogen consumption per gram of initial biomass in the first six hours of the PHA accumulation tests with continuous and pulse-wise feeding with a pulses reaching initial F/M ratios of 60 $mgCOD_{HAc}/gVSS$.

an average of 105 ± 19 s. This pulse dead time resulted in almost 1 hour out of the six hours with no exogenous substrate supply. If these dead times are too long and the endogenous substrate metabolic pools also become exhausted, then cells can stop their activity or even start to consume the produced PHA. If the next pulse is given "just-in-time" and the rate of exogenous mass transport of substrate into the cell is faster than the rate of polymer storage, then accumulation rates may be sustained despite the periodic (near) absences of exogenous substrate. On the contrary, when substrate was fed in excess by continuous feeding or in large pulses, interruptions of exogenous substrate supply are avoided. If the rate of polymer storage is not influenced by smaller or large pulse feeding then that would suggest that the inter-pulse dead time was sufficiently short to avoid repeated substrate limitation. Pulse feeding with periodic exogenous substrate limitation may result in a delay in biomass adaptation towards a growth response. The lack in such interruptions in the cases of excess (large pulse or excess continuous excess feeding) with sustained available exogenous substrate could explain why higher biomass growth and nitrogen removal rates were observed with excess feeding and few if any substrate interruptions, as observed in Figure 3.5.

### 3.3.3. PHA accumulation performance without nitrification and/or pH control

Nitrogen compounds are known to influence growth and PHA production (Johnson et al., 2010a; Fra-Vázquez et al., 2019). In addition, nitrifying bacteria, if present, would compete with the PHA-storing bacteria for ammonium, nitrite, and oxygen and can account for 25% of the overall oxygen consumption in activated sludge (Estévez-Alonso et al., 2021b). As expected, in those accumulation tests where nitrification was not inhibited, an average of 20% higher oxygen consumption was measured. However, the PHA-storing bacteria themselves were not affected by the presence of active nitrifying bacteria. PHA contents and yields on substrate were similar to those obtained in control experiments with ATU addition and pH control, as observed in Figure 3.2 and 3.3. This is likely due to the non-limiting dissolved oxygen concentrations. Even though nitrification was active, dissolved oxygen values were not lower than 1 $mgO_2/L$. Oxygen limitation is expected for concurrent PHA production and nitrification below this value (Estévez-Alonso et al., 2021b). Co-existence of PHA-storing bacteria and active nitrification in enrichment reactors have been previously reported (Fra-Vázquez et al., 2019). Also in this case, active nitrification did not affect the enrichment of PHA-storing biomass. In industrial mixed culture PHA production, oxygen supply is expected to determine the maximum process volumetric productivity. Oxygen should preferentially be for PHA production and not for other metabolic processes in order to maximize volumetric productivities. Thus, nitrification needs to be minimized. For this purpose, several strategies that rely on the use of low dissolved oxygen concentrations ($< 2$ $mgO_2/L$) to limit the growth of nitrifying bacteria are available in literature (Estévez-Alonso et al., 2021b). These lower dissolved oxygen concentrations have a more negative effect on nitrifying than in PHA-storing bacteria.

A high salinity level can have a negative influence on bacterial growth and PHA production (Palmeiro-Sánchez et al., 2016b). In the present work, the feedstock addition and the use of pH control resulted in the addition of 3.1-4.2 $gK^+$ and 3.5-4.7 $gCl^-$. To reduce the concentration buildup of salts in the reactor, experiments without pH control were performed. The obtained pH profile was different from previous experiments. Initial pH values ranged between 7.5 to 8.0, but when

pH was not controlled, it slowly increased towards pH 9.0. Despite a lower total amount of salts added to the reactor when pH was not controlled, results of PHA content and yields on substrate were similar, as observed in Figure 3.2 and 3.3. These results do not support what has been previously reported (Liu et al., 2011; Serafim et al., 2004). Higher PHA contents and yields have been reported when pH was not controlled or was controlled at pH values between 8 and 9. When pH was not controlled, initial pH was between 7 and 9 and final pH ranged from 8 to 9. Nonetheless, the highest PHA content ever reported with mixed cultures was obtained when pH was controlled to a value of 7 ± 0.1 (Johnson et al., 2009a). From the results obtained in this work, it can be concluded that at industrial PHA production with waste activated sludge, the use of pH control can probably be avoided, and consequently process costs due to amounts of chemicals added for pH control can be avoided.



### 3.3.4. PHA accumulation performance with a biomass acclimation step

A biomass acclimation step prior to the PHA accumulation has been shown to promote higher accumulation degrees in PHA production by municipal activated sludge (Morgan-Sagastume et al., 2019; Bengtsson et al., 2017b). In the present work, higher PHA contents were obtained when the biomass was subjected to three short feast and famine cycles. PHA contents in tests with biomass acclimation were 0.40-0.42 gPHA/gVSS compared to only 0.28-0.37 to tests without an acclimation step. Initial PHA production rates were also higher when an acclimation step was applied, as shown in Figure 3.2. Despite the fact that higher acetate uptake rates and PHA contents were observed, average PHA yields on substrate did not change compared to control experiments. These yields were in the range of 0.30-0.34 $gCOD_{PHA}/gCOD_{HAc}$. Higher yields on substrate were obtained in previous studies (Morgan-Sagastume et al., 2019). The reason why similarly high yields were not obtained in this work remains unclear. A fundamental understanding of acclimation adaptation was not part of the current work and further research is required. The proposed mechanism(s) behind this adaptation response could be related to a stimulation for growth of the PHA-storing bacteria, the waking-up of a dormant biomass fraction present in the activated sludge, or a physiological adap-

tation of the PHA-storing cells. Based on previous research, this acclimation step is understood to be a physiological adaptation or an activation of dormant cells rather than the growth of the storing biomass. An increase in the final PHA content from 0.3 to 0.4 gPHA/gVSS cannot be explained only by an increase in PHA-storing biomass. If it is assumed that the PHA-storing bacteria can accumulate a maximum of 0.6 gPHA/gVSS (Pei et al., 2022a) and the initial VSS concentration in the reactor is 2.5 gVSS, an increase in the final PHA content from 0.3 to 0.4 gPHA/gVSS, would mean that 25% more PHA-storing biomass (625 mgVSS) should have been formed in the acclimation step compared to experiments when the acclimation step was not performed. This level of biomass increase is not possible because the amount of COD added in these three cycles, only 450 mgCOD. These amounts of COD could result in the production of 165 mgVSS.. Ongoing investigation is directed to elucidate the mechanism(s) behind the acclimation response.

### 3.3.5. Polymer recovery and recovered polymer properties

Polymer properties, in the biomass and for extracted films, were evaluated for triplicate batches of the harvested, acid-washed, and dried biomass from 5 of the accumulation tests (see supplementary material). The five batches comprised four sets made with pH control and ATU addition (P-FM20+pH+ATU, P-FM60+pH+ATU, P-FM400+pH+ATU, and continuous excess feed), and one set with pH control, but without ATU addition (P-FM60+pH). The average 3HV content measured for produced PHBV in the biomass was low (1 ± 1% wt., n=15). Negligible relative amount of 3HV was expected given acetic acid as sole substrate. Ash (inorganic content) of the dried biomass solids was high (21 ± 3% g/g, n=15) and this contributed to a significant total biomass mass loading for targeted polymer extraction concentrations of 20 mgPHA/mL. Extraction biomass loading was also high due to lower biomass PHA contents on average (16 to 31 gPHA/gTS), because these accumulations were made without the acclimation step. However, extractions could still be readily made. Following an assessment of polymer properties and extraction yields, statistical evidence for any effect of polymer impurity level on assessed properties, polymer properties on extraction outcomes, and accumulation conditions on polymer properties was tested.

A consistent level of 4.1 ± 0.6% wt. of the non-polymer biomass was co-extracted. Therefore, differences in biomass mass loadings for the targeted 20 mgPHA/L polymer extraction concentration resulted in a corresponding range in variability of the total extract purity from 79 to 89% PHA. The extractable amount of PHBV was also found to be variable and ranged from 61 to 100%. The polymer melt behaviour was assessed directly in the dried biomass and for the recovered polymer film based on the second melting ramp. Melt peak enthalpy and temperature for the PHA-in biomass, and PHA-film, were 63 ± 9 J/gPHA and 175 ± 1°C, and 91 ± 9 J/gPHA and 173 ± 1 °C. For reference, the same peak enthalpy and melt temperature for Biomer PHB was 102 J/gPHA and 175°C ($M_w$ = 1325 kDa). Melt peak temperatures were similar in all cases, but melt enthalpies were lower than the commercial PHB for the recovered films, and significantly lower for the PHA-in biomass. A minor component of 3HV in the PHBV produced could lower the expected melt enthalpy compared to pure PHB. Acid-washing of the biomass before drying promoted a consistently high thermal decomposition temperature ($T_d$ = 287 ± 2°C at 10°C/min, n=15) as was expected (Chan et al., 2017). A high thermal decomposition temperature is essential for mitigating polymer molecular weight loss for high temperature drying and solvent extraction (Werker et al., 2013a,b, 2020; Bengtsson et al., 2017b). The extracted PHBV weight average molecular mass was consistently high on average but still variable from the 15 accumulation batches in the range from 1000 to 2000 kDa, as observed in Figure 3.6. This range of PHBV molecular weight is uniquely higher on average than typically reported for mixed culture PHA production (Serafim et al., 2008; Bengtsson et al., 2010; Albuquerque et al., 2011; Palmeiro-Sánchez et al., 2016b; Lorini et al., 2021a; Rodrigues et al., 2022). Higher molecular weights have been shown to result if the accumulated PHA-rich biomass, after acid-washing, is dried by first heated rapidly from ambient conditions (Werker et al., 2020, 2013b). An increase in heating rate was achieved by disposing the biomass to a higher initial oven temperature (120°C for 30 minutes and then drying at 105°C overnight).

There was no correlation to suggest that the different relative levels of co-extracted non-polymer biomass, that influenced extracted polymer purity, affected measured melt properties or molecular weights by solution rheology. In-

terestingly, the concentration of dissolved non-polymer biomass in the solvent after extraction was correlated to both the concentration of extracted polymer (P=0.0112) as well as the mass of non-polymer biomass loaded into the tube for the extraction (P<0.0001). Multi-linear regression suggested that 1 mg/gDMC of non-polymer biomass used in the extraction would result in 0.03 mg/gDMC dissolved biomass impurity. Additionally, every mgPHA/gDMC extracted polymer carried an estimated amount of 0.07 mg/gDMC impurity into solution in this particular study. Even if co-extracted non-polymer impurities can be removed after extraction, the challenges and costs to reach a polymer product with high purity (> 98%) would be reduced if the polymer content in the biomass is higher.

Extraction recoveries were lower than expected based on previous experience with similar dried biomass having higher biomass PHBV contents. The concentration of PHBV that became dissolved in the DMC was naturally a function of how much biomass containing PHBV was loaded to the extraction system (P<0.0001). It was predicted that on average for every mgPHA/gDMC loaded to the extraction tube, 0.8 mgPHA/gDMC became dissolved in solution. However, it was also found that the extraction recovery was correlated to the expressed level of melt (or crystallization) enthalpy (P=0.0002). The melt enthalpy of the PHA in the biomass was on average 70% of the level for the same polymer extracted from the biomass. Bacteria store PHA as granules (so-called carbonosomes (Jendrossek and Pfeiffer, 2014)), and the polymers within in-vivo granules are maintained in a hydrated non-crystalline state enabling metabolic activity for polymer storage and its mobilization. These observed in-situ melt property dependent extraction recoveries suggest that the more the polymer in the biomass expresses thermoplastic behaviour as a polymer, the more it can be readily extracted. Therefore, methods of downstream processing before solvent extraction should work to maximize that the PHA coalesces (Sedlacek et al., 2019) to behave more as a thermoplastic polymer and less like its native carbonosomes state in-vivo.

Extraction recovery was also negatively correlated to the recovered polymer molecular weight (P=0.0014). The negative influence of molecular weight on extraction recovery introduces a practical consideration for commercial developments. Diffusion of solvent, dissolving the polymer, and polymer transport into

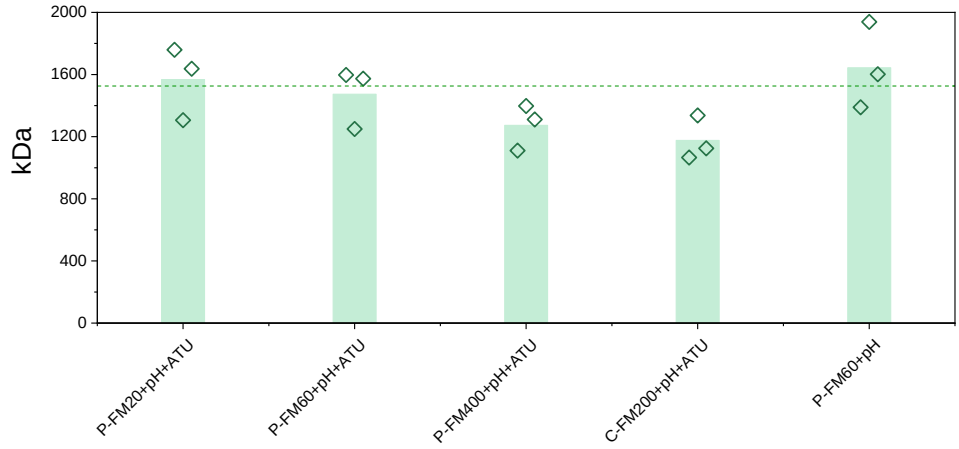


Figure 3.6. Overview of PHA molecular weights obtained at the end of the PHA accumulation tests with continuous excess feeding and pulse-wise feeding and subsequent standardized polymer extraction. Dash line indicates the molecular weight average value for the pulse feeding without excess substrate (P-FM20 and P-FM60).

solution are likely to be negatively influenced by increased solution viscosity and a greater tendency for gel formation that could block the biomass particle pore spaces. Higher molecular weight polymers may offer unique opportunities for certain kinds of applications (Bengtsson et al., 2017b). However, higher molecular weight can, according to these results, reduce extraction performance. That decease would decrease commercial productivity at an industrial scale. Engineering to modulate molecular weight during recovery to be as high (or low) as absolutely required for the polymer intended application is a necessary aspect for balance in value from quality and quantity at industrial scale. The standardized extraction methods applied in the present work can be adapted and applied for assessments for tuning industrial scale conditions.

The observed molecular weight variability could have been influenced by effects of unforeseen/uncontrolled differences during drying and extraction, and/or in the accumulation operations. The accumulation results were grouped between pulse feeding methods (P-FM20, and P-FM60 feeding), and excess feeding methods (P-FM400, continuous excess feeding). Excess feeding methods resulted in statistically significantly lower yield of PHA on substrate, and lower weight average molecular mass. This correspondence suggests that better yield on substrate

promotes conditions for generating polymers with higher molecular weight. It is considered that if the bacteria divert less carbon to polymer storage (excess feeding method outcomes), then the carbon flux for polymer formation will not be at its maximum possible rate. Loss of flux for rate limitation in polymer formation kinetics has been speculated to increase the inherent chain termination probability for polymerization (Werker et al., 2013a). Increase in the chain termination probability results in polymer chains with lower average molecular weight. Indications that lower yields lead to lower polymer molecular weights may nevertheless be coincidental and so these insights require continued systematic evaluations. Neglecting the excess feeding method outcomes, the molecular weight for the pulse feeding results was 1536 ± 221 kDa. This level sets a higher expected standard for what mixed culture PHA production should be able to consistently achieve. At the same time, the underlying causes for the unexplained variability of ± 221 kDa in molecular weight outcomes remains a challenge to understand further in the ongoing developments.



## 3.4. Conclusions

Waste activated sludge can be directly used for PHA production. An acclimation step combined with pulse-wise feeding strategy resulted in maximum PHA contents of 0.40-0.42 gPHA/gVSS and product yields of 0.30-0.34 $gCOD_{PHA}/gCOD_{HAc}$. Nitrification and pH control did not have an observable effect on the PHA production capacity of the biomass. PHAs with molecular weights in the order of 1500 kDa were obtained. Polymer molecular weight and thermoplastic properties in the dried biomass influenced polymer extraction recovery. A standard protocol for an activated sludge PHA accumulation test, including downstream processing and extraction, has been made available as supplementary material.

*To Chapter 2*

## 3.5. Supplementary material

Table 3.2. Overview of PHA accumulation, polymer extractions and properties (Part 1). Test 1 to 15 were performed with pH controlled at 7.5. Allylthiourea was added in tests 1 to 12, but not to tests 13 to 15.

| Test | F/M pulse | PHA content | | $Y_{PHA/HAc}$ | $Y_{X/HAc}$ | $Y_{O_2/HAc}$ | Extraction load | Extracted PHA | Extracted non-PHA | Extraction Yield |
|---|---|---|---|---|---|---|---|---|---|---|
| | mgCOD/gVSS | gPHA/gVSS | gPHA/gTSS | gCOD/gCOD | | | mgPHA/gDMC | | mg/gDMC | % |
| 1 | 20 | 0.34 | 0.29 | 0.34 | 0.07 | 0.43 | 19.3 | 13.3 | 2.0 | 69 |
| 2 | 20 | 0.35 | 0.29 | 0.34 | 0.03 | 0.39 | 19.2 | 13.7 | 1.7 | 71 |
| 3 | 20 | 0.34 | 0.29 | 0.36 | 0.00 | 0.35 | 10.0 | 6.8 | 0.9 | 68 |
| 4 | 60 | 0.30 | 0.24 | 0.38 | 0.14 | 0.52 | 19.5 | 19.1 | 3.1 | 98 |
| 5 | 60 | 0.28 | 0.23 | 0.37 | 0.14 | 0.48 | 20.0 | 15.2 | 2.7 | 76 |
| 6 | 60 | 0.27 | 0.22 | 0.31 | 0.13 | 0.52 | 19.7 | 18.0 | 3.2 | 91 |
| 7 | 400 | 0.25 | 0.20 | 0.21 | 0.01 | 0.30 | 19.7 | 16.4 | 3.3 | 84 |
| 8 | 400 | 0.23 | 0.18 | 0.22 | 0.13 | 0.39 | 19.3 | 15.0 | 3.5 | 78 |
| 9 | 400 | 0.22 | 0.17 | 0.23 | 0.18 | 0.50 | 19.1 | 14.0 | 3.2 | 74 |
| 10 | 200 | 0.22 | 0.16 | 0.19 | 0.19 | 0.58 | 18.1 | 18.0 | 4.3 | 100 |
| 11 | 200 | 0.20 | 0.16 | 0.19 | 0.10 | 0.35 | 18.2 | 11.0 | 3.0 | 61 |
| 12 | 200 | 0.25 | 0.20 | 0.24 | 0.12 | 0.53 | 20.6 | 16.3 | 3.6 | 79 |
| 13 | 60 | 0.28 | 0.22 | 0.33 | 0.06 | 0.50 | 21.7 | 17.7 | 3.2 | 82 |
| 14 | 60 | 0.35 | 0.31 | 0.28 | 0.00 | 0.52 | 20.1 | 17.0 | 2.0 | 85 |
| 15 | 60 | 0.28 | 0.27 | 0.33 | 0.06 | 0.51 | 22.1 | 14.4 | 3.3 | 65 |

Table 3.3. Overview of PHA accumulation, polymer extractions and properties (Part 2). Test 1 to 15 were performed with pH controlled at 7.5. Allylthiourea was added in tests 1 to 12, but not to tests 13 to 15.

| Test | F/M pulse | Purity | Td | [n] | Mw | 3HV content | ΔHm (in biomass) | ΔHm (Extract) | Tm (in biomass) | Tm (Extract) |
|---|---|---|---|---|---|---|---|---|---|---|
| | mgCOD/gVSS | % | °C | dL/g | kDa | %wt. | J/gPHA | J/gPHA | °C | °C |
| 1 | 20 | 87 | 295 | 2.28 | 1637 | 1.9 | 65 | 80 | 176 | 175 |
| 2 | 20 | 89 | 295 | 1.93 | 1306 | 0.5 | 56 | 85 | 176 | 174 |
| 3 | 20 | 88 | 296 | 2.40 | 1759 | 0.7 | 62 | 96 | 177 | 175 |
| 4 | 60 | 86 | 296 | 2.21 | 1573 | 0.9 | 77 | 95 | 175 | 174 |
| 5 | 60 | 85 | 296 | 1.86 | 1250 | 1.0 | 59 | 79 | 175 | 173 |
| 6 | 60 | 85 | 295 | 2.24 | 1597 | 1.5 | 77 | 114 | 174 | 173 |
| 7 | 400 | 83 | 297 | 1.93 | 1311 | 1.5 | 59 | 90 | 174 | 172 |
| 8 | 400 | 81 | 279 | 1.71 | 1110 | 1.7 | 67 | 92 | 173 | 172 |
| 9 | 400 | 81 | 296 | 2.03 | 1398 | 3.2 | 60 | 85 | 174 | 172 |
| 10 | 200 | 81 | 297 | 1.73 | 1125 | 1.6 | 70 | 86 | 173 | 172 |
| 11 | 200 | 79 | 296 | 1.66 | 1066 | 2.8 | 41 | 85 | 172 | 170 |
| 12 | 200 | 82 | 296 | 1.96 | 1337 | 1.1 | 64 | 98 | 174 | 172 |
| 13 | 60 | 85 | 295 | 2.24 | 1602 | 1.2 | 65 | 88 | 175 | 173 |
| 14 | 60 | 89 | 296 | 2.02 | 1389 | 0.5 | 69 | 98 | 175 | 173 |
| 15 | 60 | 81 | 296 | 2.58 | 1939 | 1.1 | 61 | 91 | 176 | 174 |

## 3.6. Protocol for a PHA accumulation and extraction test



This protocol provides a summary of equipment and methods for routine evaluation of direct accumulation PHA production and its recovery from dried biomass. It is meant for experienced laboratory personnel who can make and apply own risk assessments and ensure safety. Requirements for safety in the laboratory work due to chemicals, methods or equipment are not covered. Methods of the PHA extraction involves use of solvents at elevated pressures due to heating the solvent above its boiling point in a closed vessel. Failure to undertake these protocols without suitable training, precautions, protection, equipment, or working conditions will risk to result in serious injury or damage to property. Application or adaptation of the protocol are made at own risk.

### 1. Materials

- A batch reactor or fermenter equipped with a mixing system and adequate sampling ports.
- An constant flow rate oxygen supply (compressed air or pure oxygen sources).
- A pH electrode.
- A dissolved oxygen meter.
- A temperature control system.
- A dissolved oxygen based feeding control system, as explained in the next section.
- 15 mL falcon tubes.
- Municipal activated sludge.
- The accumulation feedstock, with nutrients ratio 100:1:0.05 (COD:N:P by weight), prepared with tap water as follows: 50 g/L acetic acid, 1.91 g/L $NH_4Cl$, 109.6 mg/L $KH_2PO_4$. The feedstock pH needs to be adjusted to 4.5 with KOH pellets.

- Glass digestion tubes.
- A heater block.
- A vortex.
- A preti dish.
- A convection oven.
- 15 mL falcon tubes.
- Dried PHA-rich biomass.
- Dimethyl carbonate.



## 2. Dissolved oxygen based feeding program

The dissolved oxygen based feeding system is based on previous work (Valentino et al., 2015). Feedstock inputs are controlled from on-line monitoring of dissolved oxygen concentrations. The control strategy is divided in two parts: acclimation and accumulation. For acclimation, the biomass is subjected to three feast and famine cycles before the start of the accumulation process (Morgan-Sagastume et al., 2019). Feast conditions are generated with a pulse input to reach a maximum substrate level of 150 mg$COD_{HAc}$/L and the duration of the feast phase is monitored by dissolved oxygen concentration. The applied famine period was three times longer than the feast time. After the third famine period, the accumulation feed-on-demand process is started automatically. The acclimation and accumulation feed-on-demand process was as follows:

1. A steady state dissolved oxygen concentration is established before the beginning of the test ($DO_{ref}$).
2. For each feedstock input, the minimum dissolved oxygen level is continuously calculated ($DO_{min}$).
3. The subsequent feedstock input is automatically given in the accumulation

phase when:

$$\text{dissolved oxygen} = DO_{min} + 0.3(DO_{ref} - DO_{min}) \tag{3.4}$$

4. In the acclimation phase, a delay between pulse three times longer than the pulse duration is expected.



### 3. Sampling

1. Fill two 15 mL falcon tubes with reactor mixed liquor.
2. Acidified one the falcon tubes to pH 2 with sulfuric acid and mixed gently.
3. Centrifuge both falcon tubes at 3250 rcf and 4°C for 20 min.
4. Collect and filter (0.45 $\mu$m pore size filters) the supernatant of the non acidified sample.
5. Store the filtered supernatant for liquid analysis.
6. Collect the pellet of the non acified falcon tube and perform VSS/TSS analysis.
7. Discard the supernatant of the acidified falcon tube and collect the pellet.
8. Dry the acidified pellet at 105°C overnight and determine the PHA content.

### 4. PHA production test

1. Prepare a solution in tap water of 2.5 gVSS/L from the municipal activated sludge stock and add 20-50 mg allylthiourea/L.
2. Fill the reactor volume with the municipal activated sludge solution.
3. Start aeration (1 L air per volume of reactor) and set the heating bath to 25°C.
4. Calibrate the dissolved oxygen and pH meters.
5. Let the reactor aerate overnight or enough time to consume all the readily biodegradable COD.

6. Start the feeding program. Preferably, it is programmed in a way that the acclimation phase is performed during the night and the accumulation phase starts early in the morning.
7. Take first sample after acclimation and just before the accumulation starts (time zero). Other sampling times can be 2, 4, 6 and 24 hours.
8. Terminate the PHA accumulation test after 24 hours.
9. Collect the reactor mixed liquor and centrifuge at 3250 rcf and 4°C for 20 min.
10. Discard the supernatant and re-suspend the pellet in tap water.
11. Acidified to pH 2 with sulfuric acid.
12. Centrifuge again at 3250 rcf and 4°C for 20 min.
13. Collect the pellets and discard the supernatant.
14. Dry the pellets at 120°C for 30 min followed by 105°C overnight.
15. Collect the dried pellets.

### 5. PHA extraction test

1. The polymer is extracted in 13 mL dimethyl carbonate.
2. The theoretical polymer concentration is 20 mgPHA/mL.
3. Grind the dried pellet.
4. Estimate the PHA content of the dried pellet.
5. Introduce 260 mgPHA (in dried biomass) in the glass digestion tube.
6. Re-dry for 30 min.
7. Add the 13 mL dimethyl carbonate to the glass digestion tube and close them with sealed caps.

8. Vortex and place them in a 140°C pre-warmed heater block.
9. Perform a 20 min extraction and vortex every 5 min.
10. Transfer the digestion tubes to a 80 pre-warmed heater block.
11. Let the dried biomass settle.
12. Carefully decant 11 mL of the solvent solution to pre-warmed (90°C ) 15 mL Falcon tubes excluding most suspended solids.
13. Centrifuge the warm solution up to 9418 x g over 2 minutes to remove suspended solids fines.
14. Carefully decant a weighed amount (about 10 mL) of the still hot solution directly into a tare weighed soda-lime glass petri dish.
15. Evaporate the solved at 85°C with gentle heating and form a dried solution cast polymer film.
16. Use the dried solution cast polymer film to perform the desired analysis (PHA content, thermal properties, molecular weight, HV content)

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# 4

# Exploring limits of PHA production by municipal activated sludge

This chapter has been published as Pei, R.[1], **Estévez-Alonso, Á.**[1], van Loosdrecht, M.C.M., Kleerebezem, R., Werker, A., 2022. *Exploring the limits of polyhydroxyalkanoate production with municipal activated sludge.* Environmental Science & Technology, **56**, (16), 11729–11738 [1]Pei, R. and Estévez-Alonso, Á. contributed equally.

## Abstract

*Municipal activated sludge can be used for polyhydroxyalkanoate (PHA) production, when supplied with volatile fatty acids. In this work, standardised PHA accumulation assays were performed with different activated sludge to determine 1) the maximum biomass PHA content, 2) the degree of enrichment (or volume to volume ratio of PHA-accumulating bacteria with respect to the total biomass), and 3) the average PHA content in the PHA-storing biomass fraction. The maximum attained biomass PHA content with different activated sludge ranged from 0.18 to 0.42 gPHA/gVSS and the degree of enrichment ranged from 0.16 to 0.51 volume/volume. The average PHA content within the PHA-accumulating biomass fraction was relatively constant, and independent of activated sludge source, with an average value of 0.58 ± 0.07 gPHA/gVSS. The degree of enrichment for PHA-accumulating bacteria was identified as the key factor to maximise PHA contents when municipal activated sludge is directly used for PHA accumulation. Future optimisation should focus on obtaining a higher degree of enrichment of PHA accumulating biomass, either through selection during wastewater treatment or by selective growth during PHA accumulation. A PHA content in the order of 0.6 gPHA/gVSS is a realistic target to be achieved when using municipal activated sludge for PHA production.*

## 4.1. Introduction

Municipal wastewater treatment plants (WWTP) rely on the use of complex microbial communities to efficiently treat and robustly remove and/or recover carbon, nitrogen, phosphorus, as well as other selected forms of contamination from wastewater (Wagner and Loy, 2002; Kehrein et al., 2020). Different process configurations have evolved over the last century to efficiently remove these contaminants, involving nitrification- denitrification and enhanced biological phosphorus removal (Lofrano and Brown, 2010; Orhon, 2015). Different bioprocess configurations will create environmental pressures to select for different microbial communities. These microbial communities directly relate to process functional performance (Lu et al., 2014; McIlroy et al., 2015; Cydzik-Kwiatkowska and Zielińska, 2016). For instance, biological phosphorus removal selects for polyphosphate accumulating bacteria and nitrification-denitrification selects for denitrifying heterotrophic bacteria. Selective pressures can be due to alternation of anaerobic, anoxic and aerobic process stages where different types of microorganisms may use different kinds of carbon sources, electron donors, electron acceptors, and/or energy sources present in the influent wastewater. Due to these alternating conditions, WWTPs often impose very dynamic environments for microorganisms and often harbour an enormous diversity of microorganisms.

Dynamic environments also tend to enrich for microorganisms that are able to store intracellular compounds as carbon or energy reservoirs. Such storage allows for effective growth in dynamic environments. For example, polyphosphate, glycogen and/or polyhydroxyalkanoates (PHA) may be accumulated by certain species of bacteria (Van Loosdrecht et al., 1997; Majone et al., 1999). Polyphosphate and glycogen storing microorganisms are capable of using intracellular polyphosphate and glycogen pools as energy sources to take up and store external substrate in the form of PHA in the absence of an electron acceptor. PHA is normally produced intracellularly as energy and carbon storage polymers to deal with the alternating presence and absence of carbon source or electron acceptor (Van Loosdrecht et al., 1997; Mino et al., 1998; Seviour et al., 2003). Most aerobic and anoxic bacteria can store PHA as carbon and energy reservoirs when organic carbon is available but other nutrients for microbial growth are (temporarily) missing.

The stored PHA can be used for microbial growth when no external organic carbon source is available, but all other intra or extracellular growth factors are sufficiently present.

PHAs stored in biomass can be recovered as biodegradable polymers with thermoplastic and mechanical properties of interest for bioplastic formulations and industrial applications (Kourmentza et al., 2017; Estévez-Alonso et al., 2021a). The surplus activated sludge produced in municipal WWTPs, can be a biomass resource to produce PHA, if a suitable feedstock rich in volatile fatty acids, is supplied to this biomass in a PHA accumulation process (Beun et al., 2000b; Bengtsson et al., 2017b). The direct use of waste activated sludge to produce PHA, without further enrichment, has been widely studied in recent years at lab and pilot-scales (Kourmentza et al., 2017; Estévez-Alonso et al., 2021a). However, the reported maximum achieved PHA contents with waste activated sludge have been lower on average than those obtained when a PHA producing enrichment culture is used, 0.4-0.6 gPHA /gVSS compared to 0.4-0.9 gPHA/gVSS. One reason for lower observed PHA content is anticipated due to an expected lower fraction of PHA-accumulating bacteria in municipal activated sludge. Still, the achieved PHA contents are higher than 0.4 gPHA/gVSS, which has been reported to be the minimum threshold for making a economical viable PHA production process (Bengtsson et al., 2017b).

Determination of the biomass (average) PHA content is most commonly reported on a mass basis (Reis et al., 2003; Kourmentza et al., 2017), as grams of PHA with respect to the total biomass in the sample represented by volatile suspended solids (VSS). With this metric, it is not possible to distinguish the contribution of individual populations to the total PHA production. In a recent work, a new staining method with microscopy and image analysis was developed and applied to differentiate and quantify between the PHA-accumulating and non PHA-accumulating biomass fractions in activated sludge (Pei et al., 2022c,b). With this tool, the degree of enrichment for the PHA-accumulating biomass fraction was estimated and directed towards understanding for variations in PHA production processes due to combined factors of amount of polymer stored and fraction of the biomass actively storing polymers. This created an opportunity to explore in-

trinsic characteristics of microbial cultures to be used for PHA production. With the combined evaluation of maximum biomass PHA content, and the biomass degree of enrichment, the biomass PHA content in the PHA-accumulating biomass fraction was estimated.

The aim of this work was to critically assess the PHA accumulation potential of activated sludge from different municipal WWTPs. The goal was to determine the degree of enrichment for PHA accumulating bacteria and to reveal limits for directly using surplus activated sludge as a biomass source for industrial scale PHA production. PHA accumulation potential assays for activated sludge sourced from a set of six different municipal WWTPs were assessed in combination to the biomass degree of enrichment for PHA-accumulating bacteria. Results and insights from the established standardised PHA accumulation methods together with selective complimentary staining with confocal microscopy image analyses are reported and discussed herein.



## 4.2. Materials and methods

### 4.2.1. Sludge source and feedstock

Grab samples of activated sludge from six different municipal WWTP were used for standardised PHA accumulation assays (Table 4.1). The set of WWTPs were selected based on process configuration, either nitrification and denitrification with chemical phosphorus removal (AO) or biological phosphorus removal ($A^2O$). Some WWTPs had also been evaluated in a previous study and this allowed for direct comparison of results (Bengtsson et al., 2017b). Mixed liquor samples (5 L) were obtained from the main aerobic process and were settled for 30-60 min. The supernatant was decanted and settled activated sludge was delivered on the same day to Wetsus (Leeuwarden, The Netherlands) by courier. Samples were stored at 4°C pending assays. The experimental period was between March and June 2021.

The accumulation feedstock, with nutrients ratio 100:1:0.05 (COD:N:P by weight), was prepared with tap water as follows: 50 g/L acetic acid, 1.91 g/L $NH_4Cl$, 109.6 mg/L $KH_2PO_4$. The feedstock pH was adjusted to pH 4.5 with KOH pellets.

Table 4.1. Municipal WWTPs. AO: anoxic-aerobic; $A^2O$: anaerobic-anoxic-aerobic. More information about the selected WWTPs can be found in the WAVES dashboard (https://live-waves.databank.nl/).

| WWTP | Country | Capacity (kPE) | Process | P-removal | Primary settling |
|---|---|---|---|---|---|
| Bath | NL | 536 | AO | Chemical | Yes |
| Leeuwarden | NL | 250 | AO | Chemical | No |
| Beverwijk | NL | 351 | AO | Chemical | Yes |
| Almere | NL | 329 | $A^2O$ | Biological | No |
| Dordrecht | NL | 310 | $A^2O$ | Biological | No |
| Winsum | NL | 23 | AO | Chemical | No |



### 4.2.2. PHA accumulation assays

PHA accumulation assays were over 48 h in a double-jacketed glass bioreactor (1 L working volume) at 25 ± 0.1°C. Agitation at 150 rpm was accomplished by standard three-bladed turbine (R60, CAT Scientific, Germany). pH was monitored but not controlled and ranged from 7.5 to 9.0. The airflow rate was fixed at 1 L/min (MV-302, Bronkhorst, Germany). Dissolved oxygen and pH probes (COS81D and CPS11D, Endress & Hausser, The Netherlands) were coupled to a 4-channel transmitter (Liquiline CM444, Endress & Hausser, The Netherlands) and measurements were logged every 10 s. Probes were calibrated according to manufacturer instructions for each assay. Substrate dosing diaphragm pumps (Stepdos 10, KNF, The Netherlands) were actuated by PLC (Logo! 8 and Logo! TDE, Siemens, Germany).

The standard PHA accumulation assay was performed to evaluate a PHA accumulation potential for the different municipal activated sludge sources. For each assay, gravity thickened activated sludge samples were diluted with tap water to nominally 2-3 gVSS/L and allylthiourea (50 mg/L) was added directly to the reactor to inhibit nitrification. The mixed liquor was brought to 25 °C and conditioned with constant aeration overnight to establish a baseline of endogenous microbial activity in all cases. Subsequently, an automated acclimation comprising three feast and famine cycles was applied as previously reported (Morgan-Sagastume et al., 2019). Feast conditions were generated with a pulse input to reach a maxi-

mum substrate level of 150 mgCOD/L and the duration of the feast was monitored by changes in respiration based on dissolved oxygen trends. The famine period was dynamically adjusted to be three times longer than each respective feast time. The duration of each feast/famine cycle was dependent of the activated sludge used and it ranged from 1 to 3 h. Trends in respiration were used to estimate the oxygen mass transfer coefficient ($k_L a$). After the third famine period, the accumulation assay was started automatically. Accumulation was driven with the same feast influent pulses and control logic, but now without any famine period between pulses. Pulse inputs were controlled from on-line monitoring of dissolved oxygen according to Valentino et al. (2015).



### 4.2.3. Biomass staining and microscopy image analysis

Mixed liquor grab samples were taken at selected time points during the PHA accumulation assays. Samples were fixed with formaldehyde to a final concentration of 3.7%, and preserved in a 1:1 ratio mixed with 1X Phosphate-buffered saline and pure ethanol before storing at -20 °C. The staining of PHA and non-PHA biomass was performed with BODIPY 493/503® (BODIPY) (Thermo Fisher Scientific, MA, USA) in combination with Sypro™ Red (Thermo Fisher Scientific, MA, USA), according to Pei et al. (2022b). Fixed sample aliquots of 5 $\mu$L were loaded in reaction wells (10 per glass slide). Reaction wells were further provided with 0.5 $\mu$L BODIPY at 2 ng/$\mu$L and 0.5 $\mu$L of 100 times diluted Sypro Red. The glass slides were dried at 46 °C. Residual dye was rinsed from the dried slide with Milli-Q water and slides were then dried again with compressed air before mounting with VECTASHIELD® HardSet™ Antifade Mounting Medium H-1400-10 and sealing.

The fixed and stained samples were evaluated by a Confocal Laser Scanning Microscope LSM 880 (Carl Zeiss, Germany) with Plan-Apochromat 63x/1.4 Oil DIC objectives (Carl Zeiss, Germany). Methods of image capture were as described in Pei et al. (2022b). Each reaction well was first surveyed to get an overall impression. Then, images from 10 randomly selected fields of view containing floc structures were acquired. For each field of view, BODIPY and Sypro Red were excited with Argon laser (488 nm) and a DPSS 561-10 laser (561 nm), respectively. Overlay images were captured into separate image channels. For each channel, 16 scans were

averaged and stored at 16-bit depth. Conditions of laser power and gain were conserved in the set of 10 images (2 channels per image), and imaging conditions were otherwise kept similar from well-to-well.

Images were evaluated in Fiji Image J (ImageJ2, Ver 1.52P). For each set of images, brightness was maximized, without overexposing for individual pixels, and the cut off for background threshold intensity level was established by visual inspection. Total pixel counts representing PHA and protein (non-PHA biomass) volumes in the plane of focus for activated sludge flocs in each field of view were measured.



For each field of view, the relative area ratio for PHA to non-PHA biomass ratio (v/v) was calculated:

$$\text{PHA to non-PHA biomass ratio (v/v)} = \frac{\text{PHA Area}}{\text{Protein Area}} \tag{4.1}$$

The average ratio from 10 fields of view represented the estimated ratio of PHA to non-PHA biomass (v/v) for each well. The activated sludge degree of enrichment was defined as the average PHA to non-PHA biomass ratio (v/v) that was reached by the end of the accumulation assay.

### 4.2.4. Analytical methods

PHA accumulations assays outcomes were assessed with online logged measurements (DO, pH and temperature) and with solids and liquid analyses from grab samples of mixed liquor at selected time points in replicates of 3x 15 mL. Suspended solids were separated by centrifugation (3250 rcf and 4°C for 20 minutes). The supernatant was stored at -20°C pending liquid analyses after membrane filtration (0.45 $\mu$m pore size filters). The suspended solids pellet dry and ash weights were estimated based on Standard Methods for solids analyses (Clesceri et al., 1999). Total and volatile suspended solids (TSS and VSS) concentrations were then estimated with respect to the 15 mL sample volume. Acetic acid concentration was determined by ultrahigh pressure liquid chromatography and ammonium, nitrite, nitrate and phosphate concentrations were determined by ion chromatography, as previously reported (Estévez-Alonso et al., 2021b).

One of the 15 mL aliquots was used for PHA determination. The liquid vol-

ume was directly acidified to pH 2 with 37% HCl. The acidified suspended solids were thoroughly mixing for 5 min and centrifuged (3250 rcf and 4°C for 20 min). The harvested pellet was dried at 105°C overnight and ground. Average biomass PHA content was estimated by thermogravimetric analysis (TGA) as previously reported (Chan et al., 2017).

### 4.2.5. Data analysis

All measured parameters were corrected for effects of sample withdrawal and feedstock addition from liquid and mass balance considerations (Johnson et al., 2009b). The biomass PHA content that was measured as a function of time was expressed as mass fraction of the volatile suspended solids (gPHA/gVSS). Active biomass ($X_a$) was estimated as VSS minus PHA mass. Active biomass was assumed to be represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980). The trend for accumulated biomass PHA content was represented by least squares regression to the empirical function as in Bengtsson et al. (2017b):

$$f_{PHA} = A_0 + A_1\left(1 - e^{-k_1 t}\right) \tag{4.2}$$

where $A_0$ is the theoretical initial PHA content, $A_1$ is the theoretical maximum PHA content and $k_1$ is a constant that enabled estimation of rates as a function of time and comparison of performance for different activated sludge sources. The accumulation time constant $\tau$ ($\tau = 1/k_1$ (h)) represented process first order kinetics in reaching a maximum level of PHA content. Initial and average specific production/consumption rates and PHA yields on substrate were estimated for process times of $0.2\tau$ and $3\tau$, respectively. The times of $0.2\tau$ and $3\tau$ were when biomass reached 18% and 95% of maximum PHA content, respectively. In assays where $3\tau$ was longer than the accumulation assay period, yields and rates are reported for the last sampling time instead. The average PHA yields on substrate were calculated on a COD-basis assuming poly(3-hydroxybutyrate) (1.67 gCOD/gPHB) produced on acetate (1.07 gCOD/gHAc) added. Average specific production and consumption rates were calculated from the the cumulative amounts of acetic acid, PHA, biomass and oxygen consumed with respect to estimated active biomass levels (gCOD/g$X_a$/h).

The trends of PHA to non-PHA biomass ratio (v/v) as a function of time could also be similarly fitted by least squares regression analysis to the first-order rate equation:

$$\text{PHA to non-PHA biomass ratio (v/v)} = B_0 + B_1\left(1 - e^{-k_2 t}\right) \tag{4.3}$$

where $B_0$ is the theoretical initial ratio, $B_1$ is the theoretical final ratio, and $k_2$ is a constant that characterized the development of PHA distribution in the biomass as the PHA to non-PHA biomass ratio (v/v) for the different activated sludge sources.



The average PHA content for the fraction of the PHA-accumulating biomass in the activated sludge was determined by:

$$\text{Average } f_{\text{PHA}} \text{ in PHA accumulating fraction} = \frac{f_{\text{PHA}}}{f_{\text{PHA}} + \text{DE} \cdot X_a} \tag{4.4}$$

where DE is the degree of enrichment defined. DE was estimated by level of PHA to non-PHA biomass ratio (v/v) that evolved by the end of the accumulation assay.

## 4.3. Results

The outcomes for the standardised PHA accumulation assays from the six sources of municipal activated sludge are summarized in Table 4.2. From these assays, the degree of enrichment for PHA-accumulating biomass fraction for the activated sludge was determined results are given in Table 4.3.

### 4.3.1. PHA accumulation performance

In general, PHA levels increased over the course of the accumulation assay and asymptotically approached a maximum level of biomass PHA content. The measured plateau PHA contents were typically reached between 24 and 60 hours in most cases, and levels remained constant for the remaining duration of the assays (Figure 4.1). The maximum biomass PHA contents ranged from 0.18-0.42 gPHA/gVSS with an average value of 0.30 ± 0.08 gPHA/gVSS. When clustered by the type of WWTP configuration, AO WWTPs showed an average biomass PHA content of 0.33 ± 0.07 gPHA/gVSS (n=4) while $A^2O$ WWTPs had an average of 0.25

Table 4.2. Summary of the PHA accumulation assay results. $q_{HAc}$ stands for acetate biomass uptake rate and $Y_{PHA,HAc}$ stands for average yield of PHA produced on acetate fed. $0.2\tau$ and $3\tau$ were when biomass reached 20% and 95% of maximum PHA content, respectively.

| WWTP | Max. $f_{PHA}$ | $\tau$ | $q_{HAc}^{0.2\tau}$ | $q_{HAc}^{3\tau}$ | $Y_{PHA,HAc}^{0.2\tau}$ | $Y_{PHA,HAc}^{3\tau}$ |
|---|---|---|---|---|---|---|
| | gPHA/gVSS | h | gCOD/gX/h | | gCOD/gCOD | |
| Bath | 0.37 | 5 | 246 | 137 | 0.46 | 0.47 |
| Leeuwarden | 0.30 | 12 | 111 | 64 | 0.45 | 0.25 |
| Beverwijk | 0.42 | 17 | 142 | 108 | 0.43 | 0.24 |
| Almere | 0.18 | 7 | 84 | 47 | 0.46 | 0.31 |
| Dordrecht | 0.32 | 17 | 90 | 81 | 0.32 | 0.21 |
| Winsum | 0.23 | 10 | 141 | 51 | 0.17 | 0.16 |

gPHA/gVSS (n=2). When clustered by the presence and absence of primary treatment, WWTPs with primary treatment showed an average biomass PHA content of 0.40 gPHA/gVSS (n=2) while WWTPs without primary treatment had an average of 0.26 ± 0.07 gPHA/gVSS (n=4). Initial PHA yields on substrate were in the range of 0.4-0.7 $gCOD_{PHA}/gCOD_{HAc}$. However, the PHA yield per amount of acetate fed decreased significantly as the maximum PHA content was attained. The PHA yield on substrate diminished to levels that were below 0.10 $gCOD_{PHA}/gCOD_{HAc}$. Consequently, by the end of the accumulation assay, the average PHA yields were not higher than 0.30 $gCOD_{PHA}/gCOD_{HAc}$. This decrease indicated that there was essentially no net PHA production in the latter stages of the accumulation assays.

Some active biomass growth was observed towards the end of the accumulations, but not at the beginning. Average active biomass yields on substrate were low at the beginning of the accumulation, <0.05 $gCOD_X/gCOD_{HAc}$, but increased over time to levels in the range of 0.01-0.24 $gCOD_X/gCOD_{HAc}$. This development supports that polymer storage was more significant than active biomass growth during the initial stages of the accumulation assay. Beverwijk WWTP was a noted exception. In this case active biomass growth was observed directly from the start of accumulation. Despite observed increasing active biomass concentrations in the latter part of assays, biomass PHA contents were found to continue to increase

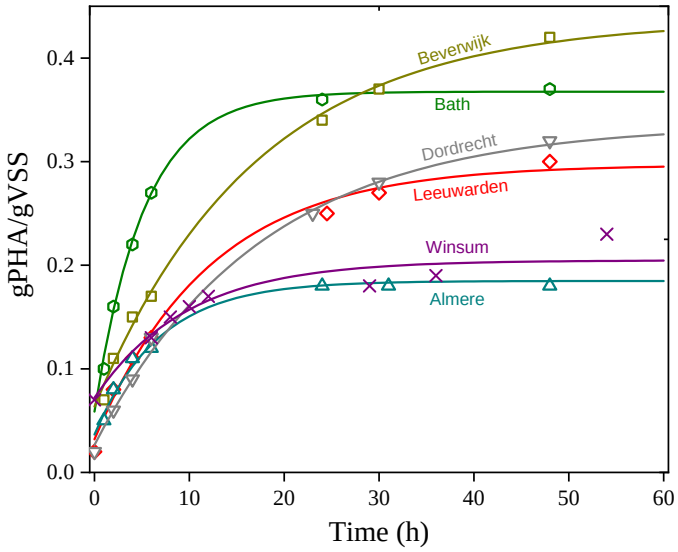


Figure 4.1. PHA accumulation trends for all the assayed WWTPs. Symbols represent the measured values and the trend lines are from Equation 4.2.

slowly (Figure 4.2). COD mass balances that were estimated from measured and estimated values could not be closed. Initially ($0.2\tau$) and at the later stages ($3\tau$ hours), only 70 ± 21 and 69 ± 23 percent of acetate as COD removed, respectively, could be accounted for as the sum of PHA and biomass produced plus oxygen consumed.

### 4.3.2. PHA distribution in activated sludge flocs

Images with the selectively stained components revealed that biomass in flocs dominated and levels of free living bacteria were considered to be relatively low. Coverage of PHA in the flocs increased on average during all the assays. However, by the end of the assays, still just a fraction of the biomass exhibited accumulated PHA, as shown in Figure 4.2. The observed morphology of the PHA accumulating bacteria was diverse for different activated sludge including rod shape, filaments and cocci. Image resolution was sufficient in some cases to observe a range of one to eight individual intracellular PHA granules per cell.

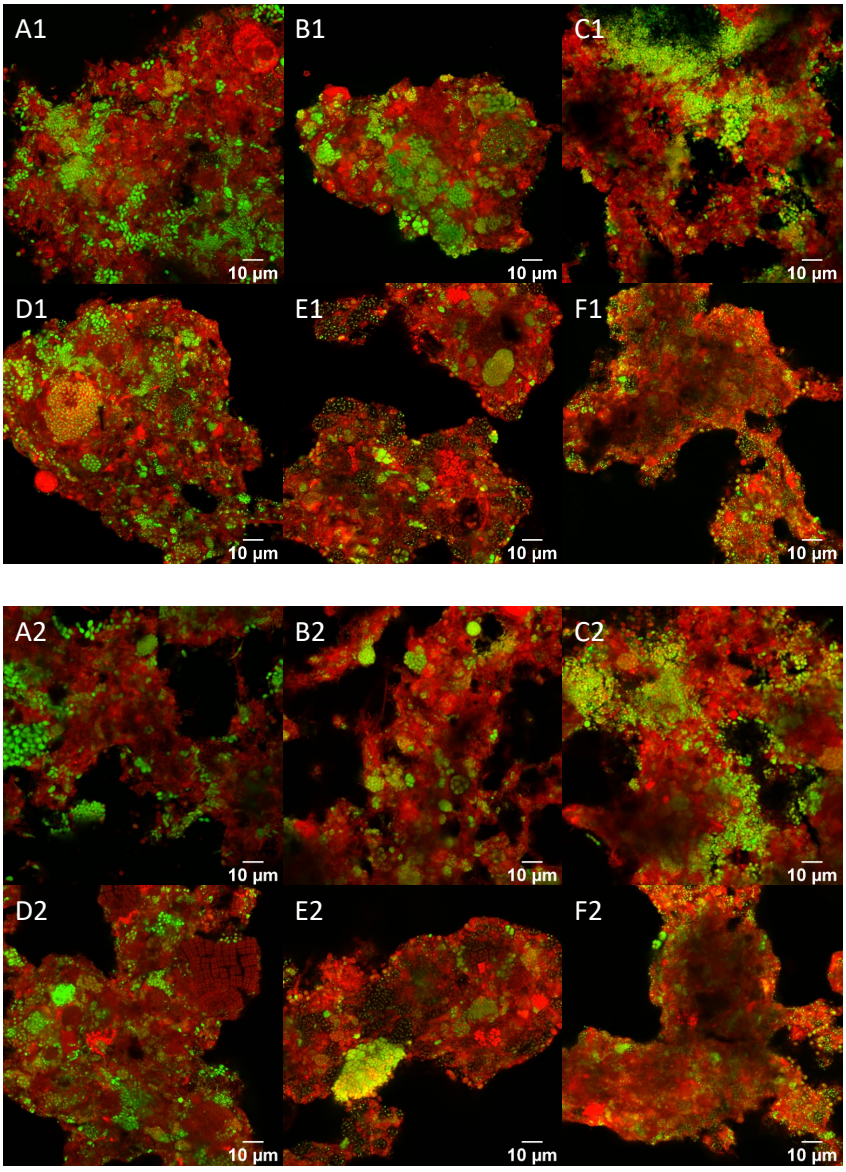


Figure 4.2. Stained PHA granules (Green) and non-PHA biomass (Red) after 48 h accumulation from WWTP of Bath (A), Leeuwarden (B), Bevewijk (C), Almere (D), Dordrecht (E) and Winsum (F) at different fields of view.

The fraction of PHA storing biomass was observed to be heterogeneously distributed within and between flocs. PHA storing activity tended to develop as aggregated clusters within individual flocs. Thus, selection for the PHA-storing phenotype was generally not considered to be uniformly distributed within the municipal activated sludge. One exception was Winsum WWTP. In this case, PHA accumulating bacteria were notably spread across observed floc volumes.

### 4.3.3. Degree of enrichment and average PHA contents in the PHA-accumulating fraction



Figure 4.2 depicts typical observations how not all the biomass was found not be actively engaged in PHA storage. The trend of PHA to non-PHA biomass average ratios (v/v) followed by analogy to trends of PHA content according to equation 4.3, as observed in Figure 4.3. The average PHA to non-PHA biomass ratio increased asymptotically towards a plateau value by 48 hours. WWTP Beverwijk was again the exception. This activated sludge exhibited a progressively increasing trend towards higher levels. These concurrent trends of average biomass PHA

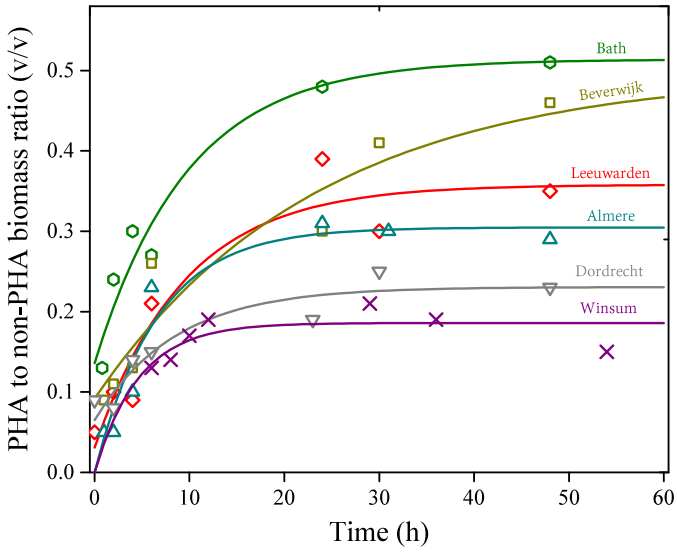


Figure 4.3. Development of PHA to non-PHA biomass ratio (v/v) during PHA accumulation assays. Symbols are the measured values from image analyses and the trend lines are from Equation 4.3.

Table 4.3. Degree of enrichment and PHA contents in the PHA-accumulating biomass fraction accumulation assays ($X_{PHA}$).

| WWTP | PHA content gPHA/gVSS | DE v/v | PHA content in $X_{PHA}$ gPHA/gVSS |
|---|---|---|---|
| Bath | 0.37 | 0.51 | 0.54 |
| Leeuwarden | 0.30 | 0.36 | 0.55 |
| Beverwijk | 0.42 | 0.42 | 0.61 |
| Almere | 0.18 | 0.31 | 0.46 |
| Dordrecht | 0.32 | 0.26 | 0.67 |
| Winsum | 0.23 | 0.16 | 0.66 |



content and degree of enrichment from six municipal activated sludge sources replicated the experience previously observed by Pei et al. (2022c,b) with activated sludge from Bath WWTP. A degree of enrichment of 1 would be indicative of a biomass with 100 percent selection of the PHA storing phenotype (Pei et al., 2022c). In the present work, with levels of less than 0.51 for degree of enrichment, not more than about half of the biomass was active in PHA storage during the assays. The estimated levels of degree of enrichment could not be readily coupled to be systematically higher or lower for either biological phosphorus removal or nitrification and denitrification WWTP process configurations. $A^2O$ WWTPs (Almere and Dordrecth) showed degrees of enrichment in the range 0.26-0.31 while AO WWTPs (Bath, Leeuwarden, Beverwijk and Winsum) ranged from 0.16-0.51.

The principal assay outcomes were the degree of enrichment and the biomass PHA content. These data enabled to estimate the average PHA content for the PHA accumulating biomass fraction (Equation 4.4). A consistently high average level of PHA storage was estimated for all activated sludge sources. The average PHA content in the PHA-accumulating biomass fraction was 0.58 ± 0.07 gPHA/gVSS. At a PHA content of 0.67 gPHA/gVSS, as observed in Dordrecht WWTP, the polymer to active biomass mass ratio is two. Thus, the PHA-accumulating bacteria from these municipal activated sludge sources exhibited similar capacities to reach up to double their organic mass as polymer.

## 4.4. Discussion

### 4.4.1. Municipal activated sludge accumulates up to 0.58 gPHA/gVSS

Microbial community-based PHA production has been widely studied over the past 20 years. However, the direct use of municipal waste activated sludge, without further enrichment, has received less research attention (Valentino et al., 2017; Kourmentza et al., 2017; Estévez-Alonso et al., 2021a). In the present work, different municipal WWTPs have exhibited different PHA accumulation potentials ranging from 0.18 to 0.42 gPHA/gVSS. These results are in line with previous experiences of PHA production with municipal activated sludge fed with synthetic feedstocks and fermented waste streams (Bengtsson et al., 2017b; Arcos-Hernández et al., 2015). These levels are still much lower than the maximum levels that have been obtained with highly enriched cultures, where PHA contents of up to 0.9 gPHA/gVSS have been attained with synthetic feedstocks (Johnson et al., 2009a). Notwithstanding, the range of PHA contents reached for enrichment cultures produced on fermented wastewater have been in the range from 0.6 to 0.8 gPHA/gVSS (Estévez-Alonso et al., 2021a). From the present investigation it is confirmed that outcomes for direct accumulation using municipal waste activated sludge are challenged by presence of non PHA-storing bacteria.

PHA accumulation patterns and PHA granules morphology were found to be diverse among the different activated sludge sources used, suggesting a high diversity of PHA accumulating microorganisms within and between different WWTPs. Even though these different microorganisms may have different respective maximum PHA contents, it was surprising to observe that, on average, the PHA content in the PHA-accumulating biomass fraction was observed to be in the range of 0.5-0.7 gPHA/gVSS. This observation also suggests that it is realistic to attain PHA content with municipal activated sludge of up to about 0.6 gPHA/gVSS. This level is significantly higher than those generally observed and historically expected with direct accumulation for waste activated sludge, and it is in line with the maximum values ever reported for the direct use of municipal activated sludge for PHA production (Chinwetkitvanich et al., 2004; Liu et al., 2011; Cavaillé et al., 2013; Arcos-Hernández et al., 2015). If the upper limits (0.6 to 0.7 gPHA/gVSS) can be consis-

tently obtained with municipal activated sludge, it would enable for much broadened generic potential to source biomass for direct PHA production. Wider generic availability of PHA producing biomass would facilitate to support PHA polymer value chains and, thereby, growth of biopolymers and chemical bio-based industrial sectors.

Why the PHA storing phenotype in municipal activated sludge accumulates an average of 0.6 gPHA/gVSS and not a higher, could not be evaluated as part of this work. A similar line of discussion is found in the literature for enrichment cultures. While highly enriched cultures have shown biomass PHA contents up to 0.9 gPHA/gVSS, not all enrichment cultures have resulted in such extraordinarily high PHA levels, and PHA contents in the range from 0.5 to 0.8 are commonly reported (Frison et al., 2015; Kourmentza et al., 2017; Crognale et al., 2019; Moretto et al., 2020b; Estévez-Alonso et al., 2021a). The experience and knowledge developments with enrichment cultures and municipal activated sludge are based on similar selection principles for the enrichment of PHA-accumulating bacteria. Dynamic process environments with alternating presence and absence of carbon source, also known as "feast and famine" have become standard practice for enrichment in this research community over 20 years (Reis et al., 2003). These selective environments exploit competitive advantage based on substrate uptake rate, that can favour PHA-accumulating bacteria due to the ability to quickly channel excess carbon in overflow metabolism (van Aalst-van Leeuwen et al., 1997). Nevertheless, feast-famine selective pressure does not necessarily enrich for superior PHA-accumulating bacteria, in absence of an intrinsic benefit to accumulate 0.9 rather than 0.6 gPHA/gVSS (Stouten et al., 2019). The experience of the specific conditions that result in enrichment of *Plasticicumulans acidivorans* or similar species of bacteria suggest that extreme levels of PHA accumulation potential are not generic to survival. Those species that reach PHA contents of 0.9 and not 0.6 gPHA/gVSS indicate that other factors govern the ability for super accumulators to dominate in certain feast famine reactors and municipal WWTPs (Stouten et al., 2019, 2021).

### 4.4.2. Degree of enrichment determines the PHA accumulation potential in municipal activated sludge

As observed in the present work, even if on average the PHA storing phenotype in municipal activated sludge can accumulate up to 0.6 gPHA/gVSS, the observed PHA content levels for the municipal activated sludge were significantly lower, ranging from 0.18 to 0.42 gPHA/gVSS. WWTPs with higher biomass PHA contents were shown to also exhibit a higher degree of enrichment. Selective pressures to enrich for the PHA accumulating phenotype in municipal WWTPs are not sufficient to drive towards a high degree of enrichment for PHA-accumulating bacteria. Different factors may affect the biomass degree of enrichment. Factors include the influent wastewater quality as well as the WWTP bioprocess configuration with its conditions of operation.



The influent wastewater is normally composed of readily biodegradable soluble COD e.g. volatile fatty acids, carbohydrates, or alcohols, and other forms of slowly biodegradable soluble and solid COD e.g. proteins, humic acids or cellulose. Bacteria can accumulate PHA using different kinds of readily biodegradable soluble COD. Nonetheless, volatile fatty acids are the preferred substrate for microbial PHA production. Other kinds of organic substrates will be directly linked to the growth of the non PHA-accumulating bacteria (Marang et al., 2018). A higher volatile fatty acids fraction in the influent wastewater will be expected to result in improved selection in the WWTP (Chua et al., 2003). However, it has also been shown that influent municipal wastewater with low levels of VFAs in the readily biodegradable fraction of the influent will support significant selection pressure (Bengtsson et al., 2017a). Further insight is required on how the readily biodegradable fraction of municipal influent wastewater can be exploited to drive the biomass towards a higher degree of enrichment.

The WWTP process configuration may affect the degree of enrichment for PHA-accumulating bacteria: the feeding pattern of the influent wastewater, the presence or absence of a primary treatment and the type of biological treatment process. How the influent wastewater is fed into the anaerobic, anoxic or aerobic zones, or to a selector or contact volume can influence development of the degree of enrichment (Krishna and Van Loosdrecht, 1999). It has been reported that

only a feast phase and not a famine period is strictly required for the enrichment of PHA accumulating bacteria (Marang et al., 2018). In another recent pilot system study, a sequencing batch reactor under feast and famine regime was used to treat municipal wastewater with low to negligible levels of volatile fatty acids. The pilot scale biomass performance for PHA production was compared to the full scale biomass. The implementation of a sequencing batch reactor enabled an idealized full-scale plug flow process with better feast conditions than the full-scale installation. This change in interpreted mixing and profile for concentrations for the pilot scale influent COD, resulted in a significant increase in the maximum PHA content to 0.49 gPHA/gVSS compared only 0.15 gPHA/gVSS for full scale activated sludge (Bengtsson et al., 2017a). This increase was assumed to be due to improved selection. If it is assumed that the PHA-accumulating fraction could accumulate an average of 0.6 gPHA/gVSS, from this work, an increase in the degree of enrichment from 0.12 to 0.64 volume to volume ratio is estimated. Deepened insight on selection pressure for municipal wastewater treatment activated sludge will require explicit coupling between configuration and operations with outcomes of the degree of enrichment methods applied in the present work.



Primary treatment is expected to lead to a higher degree of enrichment. Primary treatment can reduce the concentration of inert organic solids adsorbed in the activated sludge. Adsorbed inert solids effectively reduce the degree of enrichment of the solids. They will also hydrolyze and degrade more slowly in the process. This degradation may support growth of flanking populations of non-PHA storing microorganisms. Previously, a measurable impact of primary treatment on the maximum PHA content was not found (Bengtsson et al., 2017b). However, in the present study, WWTPs with primary treatment exhibited higher PHA contents (0.40 gPHA/gVSS) and degree of enrichment (0.47 v/v) compared to WWTPs without a primary treatment (0.26 ± 0.06 gPHA/gVSS and 0.27±0.07 v/v). These differences were statistically significant ($p < 0.05$).

The biological process configuration may influence both the degree of enrichment and biomass PHA contents. WWTPs with either AO or $A^2O$ configurations may select for different microbial communities. In the present study, and in line with previous experience, WWTPs with AO configurations had a slightly

higher biomass PHA contents and degree of enrichment (0.33±0.07 gPHA/gVSS, 0.36±0.11 v/v) compared to WWTPs with $A^2O$ configurations (0.25 gPHA/gVSS, 0.29 v/v) (Bengtsson et al., 2017b). However, the differences were not significant. Both configurations showed higher and lower biomass PHA contents. It may also be that the PHA accumulation method used in the present work is not the most suitable for polyphosphate accumulating organisms. Polyphosphate accumulating organisms are usually enriched under anaerobic/aerobic cycles, and do not only accumulate PHAs, but polyphosphate and glycogen. For $A^2O$ WWTPs, it could be of interest to start the PHA accumulation under anaerobic conditions where PHA is produced and the polyphosphate and glycogen pool are depleted, followed by a subsequent aerobic phase, as proposed previously (Bengtsson, 2009). Moreover, deepened comparative evaluations are required to understand what makes a given $A^2O$ (or AO) result in an activated sludge with higher or lower degrees of enrichment. Since both outcomes were observed, the configuration in itself was not a definitive determining factor in these cases.



Layered on top of process configuration, operational conditions including temperature and solids retention time can affect the degree of enrichment for PHA-accumulating microorganisms. Temperature has been shown to be a factor for successful enrichment of PHA-accumulating bacteria in feast and famine reactors, especially at low solid retention times (Stouten et al., 2019; Jiang et al., 2011b; Krishna and Van Loosdrecht, 1999). Higher temperatures (circa 30°C) in enrichment reactors showed a consistent response towards polymer storage, while lower temperatures (circa 20°C) showed a mix response of growth and storage. These results suggest a role of temperature on the competition between growth and polymer storage. Average annual temperatures for northern Europe are expected to be around 10°C. Outcomes for degree of enrichment, with all other factors being similar, may be different for warmer climates than The Netherlands. An influence of solids retention time on selection for degree of enrichment has not been conclusive. Some research has reported that shorter solids retention times will result in higher PHA accumulation potentials (Chua et al., 2003). However others have shown that solids retention times did not have shown an significant impact on the biomass PHA content (Sakai et al., 2015).

### 4.4.3. Strategies to maximize PHA production with municipal activated sludge

It was found that a significant fraction of municipal activated sludge from a set of northern European WWTPs comprised PHA-accumulating bacteria. Independent of the source of the activated sludge, PHA-accumulators accumulated on average in the order of 0.6 gPHA/gVSS. However, the activated sludge degree of enrichment meant that the average biomass PHA contents were lower and in the range of 0.18-0.42 gPHA/gVSS. Methods to optimize the PHA production process with municipal activated sludge need to be considered. The following methods are proposed:



1. *Before the PHA accumulation process.* The degree of enrichment can be increased before the PHA accumulation, for instance, in the municipal WWTP without the need to change the biological process by including a primary treatment or creating better feast conditions in the activated sludge process, as discussed above.

2. *In the PHA accumulation process.* The degree of enrichment may be increased directly in the PHA accumulation process if conditions for the selective growth of the PHA-accumulating biomass can be created. For Beverwijk WWTP activated sludge, PHA contents and the fraction of PHA storing biomass steadily increased over the time of the accumulation without reaching a plateau level (Figure 4.2). This observation suggests that biomass growth was selective to the PHA-accumulating biomass fraction. Examples of simultaneous growth and PHA accumulation with enriched cultures can be found in literature (Mulders et al., 2020b; Valentino et al., 2015; Cavaillé et al., 2013). However, these strategies have not been consistent in outcome, have resulted in low average PHA yields on substrate, or involved a biomass with an already high degree of enrichment. Thus, greater insight is needed to define which conditions will promote consistently for predictable concurrent selective growth and PHA accumulation during direct PHA accumulation using an activated sludge with lower starting degree of enrichment.

3. *After the PHA accumulation process.* The degree of enrichment may be in-

creased if methods are implemented for the selective removal of non-PHA containing biomass in the downstream process after the PHA accumulation. PHA and non-PHA biomass are expected to have different density. Disruption of floc structure will avail in principle a potential to separate PHA rich fractions by gradient centrifugation (Oshiki et al., 2010). Direct accumulation of municipal activated sludge was found to result in clusters of the PHA accumulating bacteria in most of the activated sludge samples. Similarly, the non-PHA biomass fraction may be selectively removed or digested. In pure culture PHA production, PHA-rich biomass has been digested by species of mealworms resulting in faecal matter of high PHA purity (Murugan et al., 2016). Similar experiments with PHA-rich biomass produced from activated sludge could be performed to test the feasibility of this approach.



*To Chapter 7*

# 5

# Calcium enhances PHA production and promotes selective growth of the PHA-storing biomass in municipal activated sludge

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## Abstract

*Activated sludge from municipal wastewater treatment processes can be used directly for the production of biodegradable polyesters from the family of polyhydroxyalkanoates (PHAs). However, municipal activated sludge typically cannot accumulate PHAs to very high levels and often low yields of polymer produced on substrate are typically observed. In the present work, it was found that the presence of calcium promotes selective growth and enrichment of the PHA-storing biomass fraction and significantly improve both PHA contents and yields. Calcium addition resulted in PHA contents of 0.60 ± 0.03 gPHA/gVSS and average PHA yields on substrate of 0.49 ± 0.03 $gCOD_{PHA}/gCOD_{HAc}$ compared to 0.35 ± 0.01 gPHA/gVSS and 0.19 ± 0.01 $gCOD_{PHA}/gCOD_{HAc}$ without calcium addition. After 48 h, three times more PHA was produced compared to control experiments without calcium addition. Higher PHA content and selective biomass production is proposed to be a consequence of calcium dependent increased levels of passive acetate uptake. Such more efficient substrate uptake could be related to a formation of calcium acetate complexes. Findings lead to bioprocess methods to stimulate a short-term selective growth of PHA-storing microorganisms and this enables improvements to the techno-economic feasibility for municipal waste activated sludge to become a generic resource for industrial scale PHA production.*

## 5.1. Introduction

Microbial community engineering aims to produce energy carriers and chemicals from waste streams using microbial communities (Kleerebezem et al., 2015). Microbial community engineering relies on the use of ecological selection principles for the enrichment of microbial communities with a desired functionality. An example of the use of microbial community engineering principles is the production of biopolymers such as polyhydroxyalkonoates (PHAs) (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021a). PHAs are naturally occurring polyesters that are produced intracellularly by bacteria to balance their growth while dealing with dynamic conditions, such as changes in substrate availability (Van Loosdrecht et al., 1997; Reis et al., 2003). In microbial community-based PHA production, PHA-storing bacteria are first enriched in a so-called *selection* reactor by applying intermittent presence and absence of carbon sources (volatile fatty acids) and/or uncoupling of organic carbon and growth nutrients supplies, namely nitrogen and phosphorus (Lemos et al., 2006; Lorini et al., 2020). Subsequently, the surplus biomass produced in the selection reactor is exploited in an *accumulation* reactor to maximize intracellular PHA content of the PHA-storing biomass (Sabapathy et al., 2020; Kourmentza et al., 2017). The maximum intracellular PHA content is constrained by the fraction of PHA-storing bacteria and the specific storage capacity of the microbial strains present in the microbial community. For instance, with enrichment cultures, where the whole microbial community can produce PHA, intracellular PHA contents of up to 0.9 gPHA/gVSS have been achieved (Johnson et al., 2009a; Jiang et al., 2011c). Notwithstanding many species of bacteria exhibit only moderate maximum storage capacity in the range from 0.5 to 0.8 gPHA/gVSS (Estévez-Alonso et al., 2021a; Sabapathy et al., 2020; Kourmentza et al., 2017). When selected microbial communities have a lower fraction of PHA-storing bacteria and therefore not all bacteria are able to produce PHAs, lower average PHA contents are observed, even if the PHA-storing bacteria that are present can accumulate up to 0.9 gPHA/gVSS (Marang et al., 2014; Crognale et al., 2019).

An example of a microbial community that exhibits a lower fraction of PHA-storing bacteria with moderate storage capacity is municipal activated sludge

(Bengtsson et al., 2017b; Pei et al., 2022b). The attraction to use of waste municipal activate sludge for PHA production is limited, in general, due to lower PHA contents in combination with lower PHA yields on substrate. Average PHA contents and yields on substrate that have been typically reported for municipal activated sludge are not higher than 0.5 gPHA/gVSS and 0.5 $gCOD_{PHA}/gCOD_{HAc}$ (Bengtsson et al., 2017b; Kourmentza et al., 2017). These values are significantly lower than those that have been obtained with specifically enriched biomass. It has been recently shown that the PHA-storing bacteria already present in municipal activated sludge systems can accumulate up to 0.6 gPHA/gVSS (Pei et al., 2022b). Thus, if strategies are developed to consistently realize such high PHA contents with municipal activated sludge, it could broaden the generic potential for biopolymer supply chains. Municipal waste activated sludge could become an ubiquitous readily available biomass source for industrial scale microbial community based PHA production.



Strategies to reach higher PHA contents with municipal activated sludge are expected to rely on methods that increase the fraction of PHA-storing bacteria in the biomass (Pei et al., 2022b). An increase in the fraction of PHA-storing bacteria will consequently result in a process that reaches the biomass maximum PHA accumulation potential. It has been hypothesised that the fraction of PHA-storing bacteria can be increased directly in the PHA accumulation process if conditions for robustly promoting selective growth of the PHA-storing biomass can be identified and applied (Pei et al., 2022b). During cell division, daughter cells contain half of the cellular PHA content of the mother cells (Pfeiffer and Jendrossek, 2012). Consequently, more PHA can be formed in parallel to cell division. In this way, PHA production and biomass growth may occur concurrently and, theoretically given the right conditions, indefinitely. A challenge is to find those conditions where these two processes can co-occur, with selective growth of PHA storing bacteria only. In pure cultures, and highly enriched open cultures, examples for simultaneous growth and PHA accumulation have already been reported (Mulders et al., 2020b; Cavaillé et al., 2016; Valentino et al., 2015; Grousseau et al., 2013). However, it is unknown if a similar strategy could be applied to diverse microbial communities that have not been specifically selected for PHA accumulation. For activated

sludge systems, Cavaillé et al. (2013) found that different degrees of phosphorus limitation promoted simultaneous growth and accumulation of PHA. Nonetheless, this strategy required a strict phosphorus level control and resulted in lower PHA yields on substrate compared to enrichment cultures.

The present work stemmed from unexpected observations made during the search for selective conditions promoting for simultaneous growth and PHA accumulation. This work concerns an investigation of effects of calcium during PHA accumulation with municipal activated sludge. At lab-scale, volatile fatty acid solutions are often used to mimic acidic fermented feedstocks. Such feedstocks are to be used for industrial scale PHA production (Bengtsson et al., 2017b). Sodium or potassium hydroxide are normally added to these feedstock solutions for pH control. In preliminary experiments, when calcium hydroxide was used instead to increase the pH of an acetic acid feedstock, it was observed that biomass growth and PHA production were positively affected. The addition of calcium in the feedstock solution induced changes that apparently created favourable conditions in the reactor for selective biomass growth concurrent to PHA production. However, since calcium addition could have influenced several water quality factors, the underlying causes for observed concurrent biomass growth with PHA production were not clear. The aim of this study was to identify the mechanism(s) behind the observed increase in PHA production and biomass growth due to calcium presence.



## 5.2. Materials and methods

### 5.2.1. Experimental set-up

PHA accumulation tests were performed over 24 and/or 48 h in a 1 L double-jacketed glass bioreactor, as previously described in Estévez-Alonso et al. (2022). Reactor temperature was 25 ± 0.1°C, pH was monitored, and air was supplied at 1 L/min. In some experiments, the pH was controlled to 8.0 ± 0.1 with 1 M NaOH solution, as indicated in Table 5.1.

### 5.2.2. Sludge source and feedstock

Activated sludge from Bath WWTP (The Netherlands) was used as the principal PHA-storing biomass for the PHA accumulation tests. The activated sludge samples were taken after gravity belt thickening and had a total solids concentration of 55 gTS/L. Validation experiments with calcium hydroxide were also performed with activated sludge from four other different Dutch WWTPs (Leeuwarden, Beverwijk, Almere and Winsum). These validation experiments were compared to outcomes from a parallel study (Pei et al., 2022b). For these other experiments, activated sludge samples were taken directly from the main aerobic process volume. The mixed liquor suspended solids were concentrated by gravity settling for 30-60 min with decanting, and had a total solids concentration of 6 to 12 gTS/L. The concentrated mixed liquor was delivered on the same day to Wetsus (Leeuwarden, The Netherlands) by courier and stored at 4°C pending experiments.



The accumulation feedstock, with nutrients ratio 100:1 (COD:N by weight), was prepared with tap water as follows: 50 g/L acetic acid and 1.91 g/L $NH_4Cl$. Phosphate levels were changed by the addition of $KH_2PO_4$, as described in Table 5.1. The feedstock was adjusted to pH 4.5 with 20 g$Ca(OH)_2$, 15.5 g$Mg(OH)_2$, 30 gKOH or 21.5 gNaOH, as described in Table 5.1. Side experiments were performed with the supplementary addition of $CaCl_2$ and KCl to the feedstock.

### 5.2.3. PHA accumulation experiments

For each assay, the concentrated activated sludge samples were diluted with tap water to nominally 2-3 gVSS/L and allylthiourea (50 mg/L) was added. The mixed liquor was brought to 25°C and conditioned with constant aeration overnight to establish a baseline of endogenous microbial activity. Subsequently, an automated acclimation was performed. Acclimation comprised three feast and famine cycles as previously reported (Morgan-Sagastume et al., 2019). Feast conditions were generated with a pulse input to reach a maximum substrate level of 150 mgCOD/L and the duration of the feast was determined from changes in respiration based on dissolved oxygen concentration trends. The famine period was dynamically adjusted to be three times longer than each respective feast time. In total, the acclimation period had a duration of 4-5 h. The trends in respiration

were used to determine the oxygen mass transfer coefficient ($k_L a$).

After the third acclimation famine period, the accumulation test was started automatically. Accumulation was driven with the same feast influent pulses and control logic, but now without any famine period between pulses. Pulse inputs were controlled from on-line monitoring of dissolved oxygen according to Valentino et al. (2015).

### 5.2.4. Analytical methods

The PHA accumulation process was monitored by online data logging (DO, pH and temperature), liquid and solids analyses. Grab samples were used for liquid and solids analyses, biomass PHA content and $CaCO_3$ determination. Suspended solids were separated from the mixed liquor by centrifugation (3250 rcf at 4°C for 20 minutes). The supernatant after membrane filtration (0.45 $\mu$m pore size filters) was stored at -20°C pending liquid analyses. Acetic acid concentration was determined by ultra-high pressure liquid chromatography and ammonium, nitrite, nitrate, and phosphate concentrations were determined by ion chromatography,



Table 5.1. Overview of the accumulation tests and feedstock solutions used.

| | Feedstock solution | | | Reactor | | |
|---|---|---|---|---|---|---|
| Experiment | Counterion | Salt | Phosphate | pH control | Duration | N° of tests |
| - | - | - | mgP/L | pH 8 ± 0.1 | h | - |
| 1 | $Ca^{2+}$ | - | 25 | No | 48 | 7 |
| 2 | $Ca^{2+}$ | KCl | 25 | No | 24 | 3 |
| 3 | $Ca^{2+}$ | - | 250 | No | 24 | 2 |
| 4 | $Ca^{2+}$ | - | 25 | Yes | 24 | 1 |
| 5 | $K^+$ | - | 25 | No | 48 | 7 |
| 6 | $K^+$ | $CaCl_2$ | 25 | No | 24 | 4 |
| 7 | $K^+$ | - | 2.5 | No | 24 | 2 |
| 8 | $K^+$ | - | 25 | Yes | 24 | 1 |
| 9 | $Na^+$ | - | 25 | No | 48 | 1 |
| 10 | $Mg^{2+}$ | - | 25 | No | 48 | 1 |

as previously reported (Estévez-Alonso et al., 2021b). The harvested biomass pellet dry weight and ash contents were estimated based on standard methods and referenced to the sample volume for total and volatile suspended solids (TSS and VSS), respectively.

The aliquot for PHA determination was directly acidified to pH 2 with 37% HCl. After thorough mixing for 5 min, suspended solids were collected (3250 rcf at 4°C for 20 min). The biomass pellet was retained and dried at 105°C. Dried pellets were ground and analyzed by thermogravimetric analysis as described previously (Chan et al., 2017). Similarly, the aliquot for $CaCO_3$ determination was directly centrifuged (3250 rcf at 4°C for 20 min) and the biomass pellet was retained and dried at 105°C. Dried pellets were ground and analyzed by thermogravimetric analysis. 5 mg of ground sub-sample were introduced to the furnace at 80°C and heated to 105°C (10 °C /min) under nitrogen atmosphere. After drying at 105°C for 10 min, the sample was heated to 550°C (10 °C/min), under nitrogen atmosphere. At 550°C, the atmosphere was switched to air and temperature was held for 30 min. After 30 min at 550°C, the sample was heated to 900°C. The weight loss trends in air were used to determine inorganic content and the calcium carbonate fraction with respect to the biomass dried total and volatile solids.



### 5.2.5. Microscopy analysis

Mixed liquor sludge samples were taken at selected time points during the PHA accumulation process. The mixed liquor was fixed with formaldehyde to a final concentration of 3.7% and preserved in a solution with ratio 1:1 of 1x PBS and pure ethanol. The fixed samples were stored at -20 °C until further processing. Fixed samples were stained with BODIPY 493/503® (Thermo Fisher Scientific, MA, USA) in combination with Sypro™ Red (Thermo Fisher Scientific, MA, USA) and were examined with confocal laser scanning microscope with statistics from fields of view, as previously described (Pei et al., 2022c). Respective overlayed stained images were acquired with a 16-bit depth in separate channels and analyzed by Fiji Image J (ImageJ2, Ver 1.52P) software. An average biomass volume-to-volume ratio of polymer to non PHA-storing biomass (v/v) was estimated (Pei et al., 2022c). This ratio was used for the determination of the fraction of PHA-storing biomass

in the microbial community.

### 5.2.6. Microbial community structure

Fresh samples were collected at selected time points from the accumulation reactor and stored at –20°C until DNA extraction. Before extraction, the samples were washed with 1x PBS solution and sonicated for 30 s. DNA was extracted from 0.5 mL mixed sample using a FastDNA® SPIN kit for soil (MPBio, USA) according to manufacturer instructions. Extracted DNA samples were purified with the kit DNA Clean & Concentrator™-5 ZYMO Research according to manufacturer instructions. DNA purity and concentrations were measured by a NanoDrop® spectrophotometer (Thermo Fisher Scientific, Germany) and a Quantus™ Fluorometer (Promega Corporation, USA), respectively. Purified DNA samples were normalized to 20 ng/$\mu$l for library preparation and 16s rRNA gene amplicon sequencing on an Illumina MiSeq at MrDNA (TX, USA). Libraries for bacteria were constructed using primers 515F (Parada et al., 2016) and 926R (Quince et al., 2011). Using QIIME2 v2019.10 (Bolyen, 2019) quality filtering and sequence data processing was applied. Sequences were demultiplexed with cutadapt (Martin, 2011). Trimming was performed after the first two bases and at lengths 190 and 200 bp for forward and reverse reads, respectively. DADA2 was used for error correction, amplicon sequence variant (ASV) inference and dereplication using default parameters, except that maxEE values were set to 4 (Callahan et al., 2016). Taxonomic assignment was performed with a naïve Bayesian classifier (Bokulich et al., 2018) trained on the curated MiDAS4 database tailored to wastewater microbial communities (Dueholm et al., 2022). The feature table and taxonomic information along with metadata were imported in R v4.0.3 using qiime2R (Bisanz, 2018) into phyloseq (McMurdie and Holmes, 2013). A reproducible workflow including all QIIME2 scripts and a R markdown document is available on Github (https://github.com/pietervanveelen/AEST_PHA_accumulation). The raw sequence data are deposited in the European Nucleotide Archive under project accession number PRJEB56263.

### 5.2.7. Data analysis

All measured parameters were corrected for effects of sample withdrawal and feedstock addition from liquid and mass balance considerations (Johnson et al., 2009b). The biomass PHA content was expressed as mass fraction of the volatile suspended solids (gPHA/gVSS). Active biomass ($X_a$) was estimated as the total VSS minus PHA mass. Active biomass was assumed to be represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980). The trend for change in PHA content ($f_{PHA}$) was represented by least squares regression to the empirical function:

$$f_{PHA} = A_0 + A_1\left(1 - e^{-kt}\right) \tag{5.1}$$



where $A_0$, $A_1$ and k are constants that allow for estimation of rates as a function of time and comparison between performances of different activated sludge samples (Bengtsson et al., 2017b). Initial and average specific production/consumption rates and PHA yields on substrate were estimated for different times. The average PHA yields on substrate are reported on a COD-basis and calculated from the amount of PHA (1.67 gCOD/gPHB) produced and substrate (1.07 gCOD/gHAc) added as a function of time. Average specific production and consumption rates were calculated based on the cumulative amounts of acetic acid, PHA, biomass and oxygen consumed with respect to the estimated active biomass levels (gCOD/g$X_a$/h).

The average PHA content in the PHA-storing biomass fraction was calculated as follows:

$$\text{Average } f_{PHA} \text{ in PHA-storing fraction} = \frac{f_{PHA}}{f_{PHA} + DE \cdot X_a} \tag{5.2}$$

where DE is the volume to volume ratio of polymer to non PHA-storing biomass by the end of the accumulation process obtained from staining and microscopy image statistical analyses (Section 5.2.5).

Microbial community analysis was based on an average of 71245 sequences per sample (range = 21463-149580), which were then filtered to retain only abundant bacterial taxa (ASVs), each representing greater than 0.05% of the total sequence abundance (i.e. retaining > 99% of sequences, 2398 ASVs). A dataset comprising

timepoints 0, 24 and 48 h was rarefied (26538 reads per sample, 100 iterations) to account for unequal sampling. Microbial community composition dynamics were visualized using principal coordinate analysis (PCoA) on the ecological Bray-Curtis dissimilarity matrix using *vegan* (Oksanen et al., 2020). A permutational multivariate analysis of variance (PERMANOVA) was applied to test the effects of feedstock and operation time on microbial biomass composition. The contributions of the 15 most dominant bacteria were depicted in the PCoA. ANOVA was used to evaluate these effects on Shannon diversity.

## 5.3. Results

### 5.3.1. Preliminary results giving rise to the work described in this paper



In preliminary tests with municipal activated sludge for PHA production, calcium hydroxide was used to increase the pH of an acetic acid feedstock solution. In these tests, a distinctly different biomass response was observed compared to experiments where KOH was used (Figure 5.1). This response was also observed in replicate experiments with four other types of municipal activated sludge, as provided in the SI. The addition of calcium hydroxide in the feedstock solution induced changes in the reactor conditions that affected the calcium and salts concentrations, phosphate availability and pH changes over the accumulation. Calcium carbonate precipitation was observed already in the first two hours of the accumulation, and therefore the concentration of soluble calcium in the mixed liquor did not increase above 500 mg$Ca^{2+}$/L over the time of accumulation. After 48 h, 50 to 55% of the total dry solids in the reactor could be identified as calcium carbonate. Calcium balances showed that the calcium added to the reactor could be found back mainly as calcium carbonate or dissolved calcium. The precipitation of calcium carbonate induced a slow, but constant decrease of pH from an initial value between 8.2 and 8.4 to a final value between 7.0 and 7.5 after 48 h of accumulation. Similarly, phosphate concentrations were below the detection limit from the start of the accumulation.

To elucidate the cause for the biomass response due to calcium added to the feedstock, deepened investigations were performed with activated sludge from

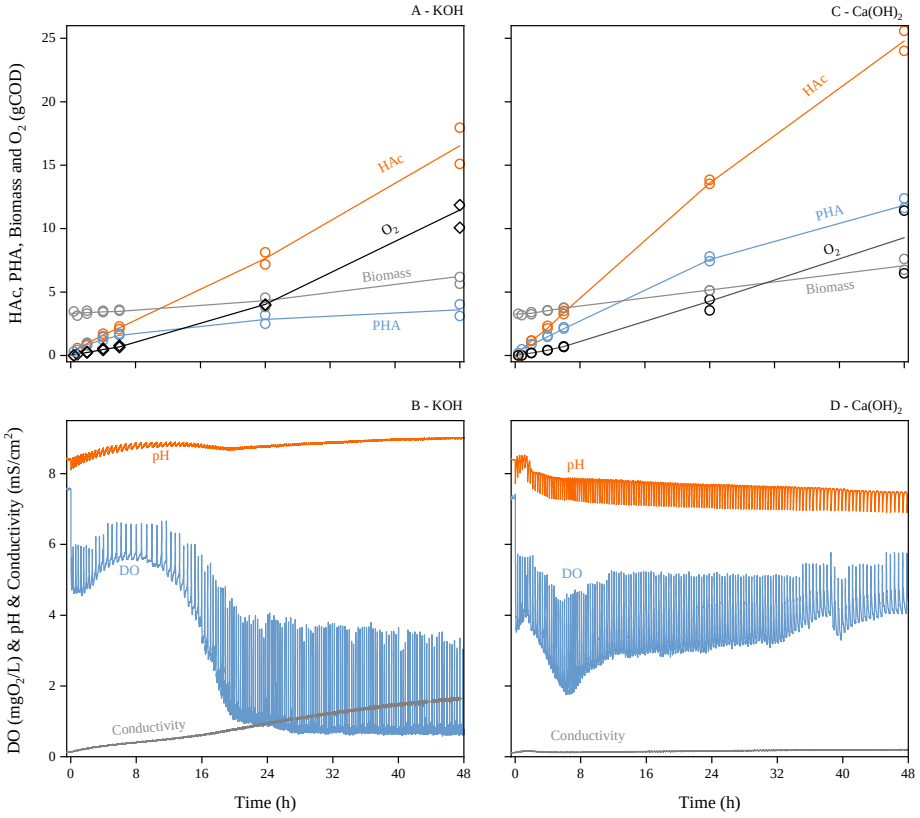


Figure 5.1. Trends of dissolved oxygen, pH, conductivity, acetate consumption, oxygen consumption, PHA production, and biomass production during PHA accumulation tests with either KOH or $Ca(OH)_2$ added to the substrate and activated sludge from Bath WWTP. For the top graphs, measured values at selected times are connected simply by straight lines to help in readability.

Bath WWTP. PHA accumulation tests were performed with $Ca(OH)_2$, $Mg(OH)_2$, KOH and NaOH to evaluate if the observed biomass response was specific to calcium. PHA accumulations tests were also performed to evaluate the potential for influences on concurrent selective biomass growth with PHA production caused by salinity, phosphate concentration, and pH control.

### 5.3.2. PHA accumulations with $Ca(OH)_2$ and KOH

A comparison of 48 h PHA accumulations tests with $Ca(OH)_2$ or KOH is provided in Figure 5.1. In the presence of calcium a higher substrate consumption was observed. This increased consumption was associated with much higher PHA production, 11.5-12.4 $gCOD_{PHA}$ compared to only 3.1-4.0 $gCOD_{PHA}$ in KOH



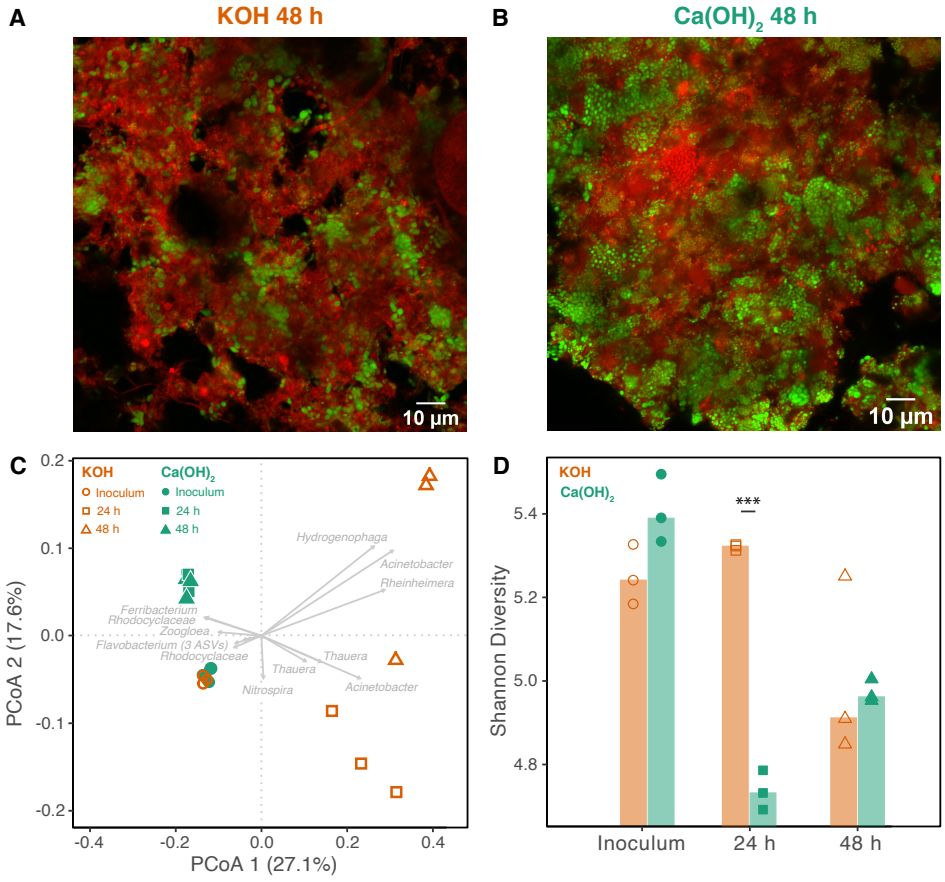


Figure 5.2. Representative microscopy images of the PHA accumulations with (A) KOH and (B) $Ca(OH)_2$ at 48 h. Red staining depicts the typical floc morphology and green stain shows the distribution of PHA granules in the biomass. (C) Shifts in microbial community in experiments with $Ca(OH)_2$ and KOH feedstocks. Principal coordinate analysis from experiments in triplicate shows predictable shifts in microbial community composition with $Ca(OH)_2$ feedstock. These shifts were driven by known PHA-storing bacteria, while different and less predictable community compositions developed with KOH feedstock. (D) Evolution of the Shannon diversity in experiments with $Ca(OH)_2$ and KOH feedstocks.

tests. Maximum biomass PHA contents in the range of 0.56-0.64 gPHA/gVSS were obtained with the addition of $Ca(OH)_2$ compared to only 0.35-37 gPHA/gVSS for KOH cases. Similarly, the average PHA yield on substrate obtained at 48 h was higher for $Ca(OH)_2$, 0.44-0.52 $gCOD_{PHA}/gCOD_{HAc}$ compared to 0.18-0.21 $gCOD_{PHA}/gCOD_{HAc}$ for KOH. Lower average oxygen yields on substrate were also observed for $Ca(OH)_2$, 0.29-0.46 $gO_2/gCOD_{HAc}$ compared to 0.65-0.69 $gO_2/gCOD_{HAc}$ for KOH (Figure 5.3). COD mass balances closed well for $Ca(OH)_2$ and KOH tests, 105 ± 6% and 102 ± 5%, respectively.

Biomass growth was slightly higher in $Ca(OH)_2$ assays, 3.4-4.3 $gCOD_X$ compared to 2.6-3.3 $gCOD_X$ in KOH tests. In $Ca(OH)_2$ accumulations, biomass growth was observed already from the beginning of the accumulation while in KOH experiments, biomass growth only started after 4-6 hours. From microscope staining, a higher fraction of PHA-storing biomass was observed at 48 h in $Ca(OH)_2$ compared to KOH, as illustrated in Figure 5.2. The fraction of PHA-storing biomass at 48 h was 0.76-0.80 (v/v) compared to 0.29-0.51 (v/v) in experiments with KOH. Despite different values in the fraction of PHA-storing biomass, the derived average PHA content within just the PHA-storing fraction of the biomass was estimated to be similar in both cases, 0.63 and 0.58 gPHA/gVSS. 16s rRNA gene amplicon sequencing analysis demonstrated that the addition of a $Ca(OH)_2$ versus KOH to the feedstock promoted differential developments of the microbial community composition, as shown in Figure 5.2 (PERMANOVA; interaction timepoint:feedstock: $F_{2, 17} = 8.4$, $P = 0.002$; Figure 5.2C). Addition of $Ca(OH)_2$ caused a rapid drop in diversity within 24 h with a shift in biomass composition that remained stable up to 48 h. This $Ca(OH)_2$ induced shift was associated with *Ferribacterium*, *Zoogloea* and other dominant *Rhodocyclaceae* genera. Conversely, addition of KOH induced a different kind of shift with retained microbial composition up to 24 h followed by a later decrease in diversity between 24 and 48 h corresponding to when most growth took place. Shifts induced by KOH addition were associated with *Acinetobacter*, *Thauera*, *Hydrogenophaga* and other dominant genera that, in contrast to $Ca(OH)_2$, continued to change in composition between 24 and 48 h.

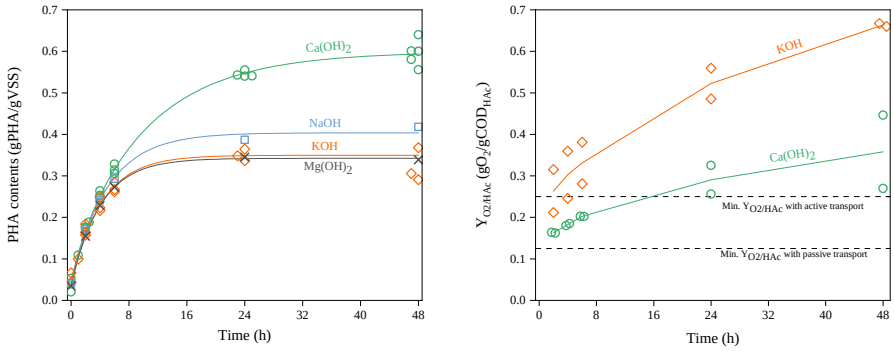


Figure 5.3. PHA content evolution in PHA accumulations with KOH, NaOH, $Mg(OH)_2$ and $Ca(OH)_2$ (left) and evolution of average oxygen yields PHA accumulations with KOH and $Ca(OH)_2$ (right).



### 5.3.3. PHA accumulations with $Mg(OH)_2$ and NaOH

To investigate if the observed effect was calcium ion, and/or cation valence specific, additional PHA accumulation tests were performed with $Mg^{2+}$ and $Na^+$ as the hydroxide counterion. $Mg(OH)_2$ and NaOH were used to raise the feedstock pH to 4.5 instead. The addition of $Mg(OH)_2$ and NaOH resulted in similar pH profiles as in experiments with KOH. Also, the process performance in terms of PHA production was very similar the KOH experiments, as observed in Figure 5.3. These results pointed to a specific effect related to, or created directly by, the presence of calcium cations during the accumulation process.

### 5.3.4. Increasing calcium concentrations in KOH experiments

To further evaluate the effect of calcium addition, accumulation experiments with KOH and extra $CaCl_2$ amounts were performed. With increasing calcium concentration, the PHA production and biomass growth increased, as observed in Figure 5.4. The test with KOH and 30 $gCaCl_2/L$ had the same dissolved calcium concentration as the feedstock with $Ca(OH)_2$ and replicated the observations of tests with only $Ca(OH)_2$. Calcium carbonate precipitation occurred from the beginning of the experiment and similar trends of pH were observed. Despite these similarities in reactor profile, the PHA production performance was lower than in

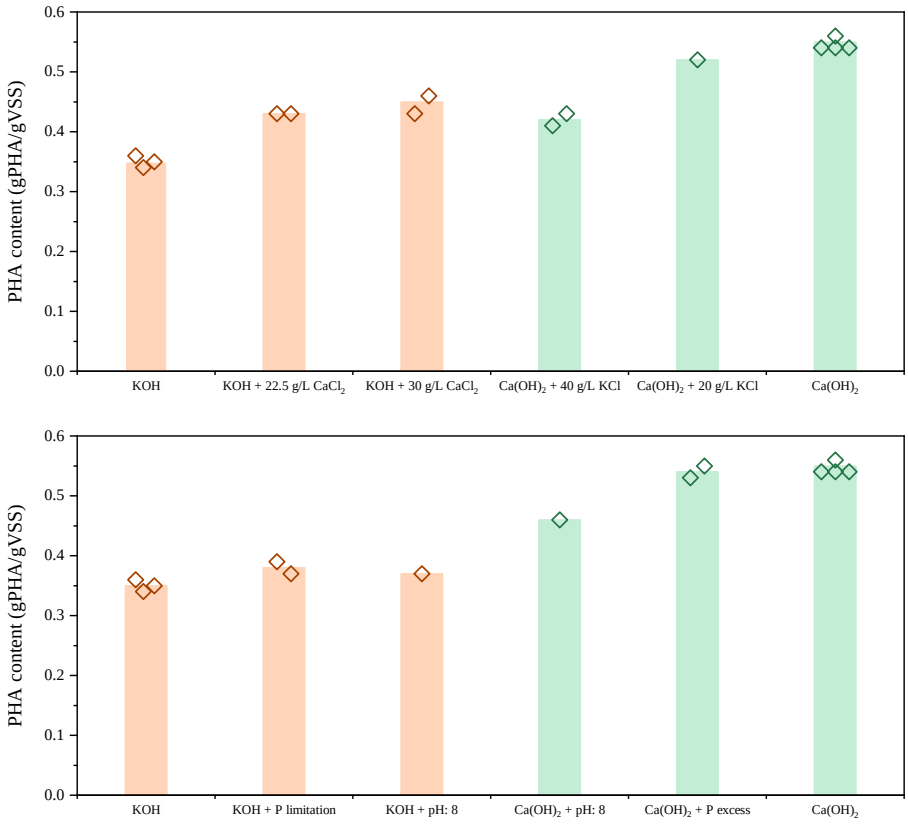


Figure 5.4. Maximum PHA contents in KOH tests with increasing calcium concentrations, $Ca(OH)_2$ tests with increasing salinity levels, $Ca(OH)_2$ and KOH tests with phosphate excess (250 mgP/L) and limitation (2.5 mgP/L) and $Ca(OH)_2$ and KOH tests with pH controlled to 8.

experiments with $Ca(OH)_2$, but still significantly higher than in experiments with only KOH. An identifiable key distinction between tests with added calcium salts, and experiments with only $Ca(OH)_2$ was the much higher increase in salinity, due to the addition of $CaCl_2$ to the feedstock.

### 5.3.5. Increasing salinity levels in $Ca(OH)_2$ experiments

The addition of $CaCl_2$ to experiments with KOH in the feedstock resulted in higher salts concentrations in the reactor compared to experiments with $Ca(OH)_2$. To evaluate the influence of salinity in $Ca(OH)_2$ experiments, control experiments

with $Ca(OH)_2$ and different KCl concentrations were performed. Similar trends were observed in all experiments, independent of the concentration of KCl in the feedstock. Calcium carbonate precipitation resulted in a progressive decrease in pH to around 7.0-7.5. Despite similarities to experiments with $Ca(OH)_2$, higher concentrations of KCl reduced the PHA production performance, as observed in Figure 5.4. Experiments with $Ca(OH)_2$ and 40 gKCl/L were analogous to experiments with KOH and 30 $gCaCl_2$/L, and similar results were obtained, as it was expected.

### 5.3.6. Phosphate excess and limitation in accumulations with $Ca(OH)_2$ and KOH

In experiments with $Ca(OH)_2$, phosphate concentrations were not detectable (< 0.33 mgP/L) in the reactor after 2-3 hours of accumulation. The addition of $Ca(OH)_2$ in the feedstock is anticipated to promote the precipitation of calcium phosphate, making the conditions phosphate limiting during most of the PHA accumulation time, notwithstanding observed biomass growth. To evaluate for an influence of phosphate limitation, control experiments were performed to evaluate KOH with phosphate limitation, as well as experiments with $Ca(OH)_2$ with phosphate excess. In these cases, no systematic differences were observed due to the imposed phosphate limitation or phosphate excess, respectively.

### 5.3.7. pH control in PHA accumulations with $Ca(OH)_2$ and KOH

The addition of $Ca(OH)_2$ in the feedstock resulted in a distinctly different pH profile during accumulations compared to KOH experiments. This difference was interpreted to be mainly due to precipitation of calcium carbonate. To allow for a more direct comparison between KOH and $Ca(OH)_2$ tests, PHA accumulation tests with pH controlled to 8 were performed, as depicted in Figure 5.4. pH control did not have an observable influence in the KOH experiments. However in the $Ca(OH)_2$ tests with pH control, lower PHA contents, PHA yields on substrate, and total PHA and biomass mass increase were observed. pH control was performed with additions from a 1 M NaOH stock solution, and 0.87 g of sodium were added to the reactor over 24 h.

## 5.4. Discussion

The combination of tests performed in this work lead to the conclusion that the presence of calcium is beneficial for selective growth of PHA-storing bacteria and PHA accumulation in waste activated sludge. Calcium presence resulted in PHA contents of up to 0.6 gPHA/gVSS, higher than previously reported. These results broaden the potential to exploit municipal waste activated sludge as a generic resource for industrial scale PHA production. With reference to the literature and by process of elimination results were examined to identify causal mechanisms for the observed influence of calcium. One explanation that could not be ruled out was the potential for formation of calcium-acetate complexes that can be more efficiently transported into the cell compared to the native acetate ion. Development of the consideration leading to this hypothesis follows.



### 5.4.1. Bacterial growth and PHA production are affected by the presence of calcium

In this work, PHA production and selective biomass growth were positively affected by the presence of calcium during accumulation. Calcium was provided due to the addition of $Ca(OH)_2$ to the feedstock used for PHA accumulation. The reproducibility of the increase in PHA production rates and yields was verified with more than ten PHA accumulations with $Ca(OH)_2$ and KOH, and with activated sludge samples obtained from different WWTPs. The increase resulted only when $Ca(OH)_2$ was added to the feedstock and could not be mimicked by the addition of KOH, NaOH or $Mg(OH)_2$, as observed in Figure 5.3. Calcium caused precipitation of mineral salts that lowered reactor phosphate concentrations and promoted a decrease in pH. These conditions were replicated by the addition of $CaCl_2$ in experiments with KOH, as observed in Figure 5.4. However, added $CaCl_2$ also resulted in a higher salinity. A negative effect of increased salinity was verified by the addition of KCl in the feedstock of $Ca(OH)_2$ tests, as observed in Figure 5.4. Even though a higher salinity did negatively influence the PHA production performance, a salt effect alone could not explain observed differences between $Ca(OH)_2$ and KOH experiments. A higher pH in $Ca(OH)_2$ experiments ($8 \pm 0.1$) also resulted

in lower PHA production rates. The addition of sodium due to pH control was not high enough to explain the decrease in performance as a consequence of salinity. This outcome suggests that the observed influence that calcium may exert is pH dependent. In the same line of reasoning from control experiments, phosphate limitation due to mineral precipitation in cases with $Ca(OH)_2$ was not found to be a cause for improved PHA production. The combination of tests and control experiments lead to the interpretation that it was the presence of calcium, directly and not the associated changes in environment created by the addition of calcium, that resulted to stimulate selective bacterial growth with concurrent PHA production right from the start of accumulation.

### 5.4.2. Calcium addition affects the cellular energy requirements and may promote passive acetate uptake



Calcium ions are known to play different roles in eukaryotic cells (Clapham, 2007). However, the role of calcium in prokaryotic cells remains unclear and research is limited (Domínguez et al., 2015). High calcium concentrations are expected to negatively affect cell activity, due to creating a high osmotic pressure. However, calcium has also been reported to benefit the stability of cell flocs and granules (Gagliano et al., 2020). More related to the current work, calcium has been reported to provoke metabolic shifts in polyphosphate accumulating microorganisms, to be involved in the formation of PHA granules, and in ion co-transport processes in the animal colon and in plant leaflets (Zhang et al., 2015; Trinidad et al., 1996, 1999; Borchert, 1986; Wolever et al., 1995).

In polyphosphate accumulating microorganisms, a direct effect of calcium was observed. An increasing calcium concentration resulted in a lower available phosphate concentration, due to calcium phosphate precipitation. Due to lower available phosphate, a metabolic shift was observed from polyphosphate to glycogen metabolism (Zhang et al., 2015). The use of glycogen slightly increased the production of PHA, with a higher content of hydroxyvalerate. In the present work, even though phosphate limitation was observed, the microbial community was not highly enriched in polyphosphate or glycogen accumulating microorganisms. This hypothesis cannot be extrapolated in general for heterotrophic bacteria and

thus, it cannot explain the results obtained in $Ca(OH)_2$ tests.

Tian et al. (2019) have suggested that calcium has a structural role in the formation of PHA granules, that are also used as calcium storage units. Calcium was found to be the most abundant cation in PHA granules from *C. necator* H16 (up to 4 $mgCa^{2+}$/gPHB) and it was proposed that calcium ions play a role in bundling the carboxyl end-groups during initial stages of the polymerization. This would imply that a minimum concentration of calcium is required to efficiently drive PHA production. In the work of Tian et al. (2019), this minimum concentration was around 25 $mgCa^{2+}$/L. In the present work, the concentration of calcium was higher than 25 $mgCa^{2+}$/L in all cases, with and without calcium addition in the feedstock. Consequently, this hypothesis can also not explain the results obtained in $Ca(OH)_2$ tests.



Alternatively, calcium may play a role in the transport of acetate into the cell. Animal colon and plant research experiences have shown evidence for calcium and short chain volatile fatty acids co-transport (Trinidad et al., 1996, 1999; Borchert, 1986; Wolever et al., 1995). In these reports, two mechanisms were given as potential explanations. The first mechanism proposes that acetate is passively transported through the cell membrane in its undissociated form. Once the undissociated acetate enters the cytoplasm, it dissociates due to higher pH in the cytoplasm compared to in the colon. The liberated proton can be excreted from the cell in exchange for an extracellular calcium ion. However, this first mechanism seems unfeasible, as the undissociated acetate can be directly used for PHA production or growth and consequently no proton can be excreted. A similar mechanism to the one proposed above is normally observed in biological reactors for phosphorus removal operated at low pH values (Smolders et al., 1994), but still it cannot explain the results observed in this work. In the current work, the reactor pH is higher than the intracellular pH, and more energy needs to be used to overcome the electric potential difference across the cell membrane. The second mechanism proposes the formation of extracellular calcium acetate complexes that diffuse through the cell membrane. Once the calcium acetate complex enters the cell, it dissociates and the calcium ion is excreted from the cell in exchange for a proton that enters the cell through the ATP-ase system. This second mechanism can potentially explain the results of the present work. Calcium acetate complexes can be formed

in aqueous solutions at pH values similar to those observed in the PHA accumulation tests (Hacht, 2008). The formation of calcium acetate complexes is affected by the soluble calcium concentration, which is also affected by the precipitation of calcium carbonate and pH. For instance, at alkaline pH, $HCO_3^-/CO_3^{2-}$ equilibrium shifts towards $CO_3^{2-}$ and calcium carbonate precipitation can occur at lower calcium concentrations. At lower calcium concentrations, the concentration of calcium acetate complexes may be too low to enable passive transport. This interpretation can explain why experiments with $Ca(OH)_2$ at pH 8 resulted in lower PHA and biomass production rates compared to experiments where pH decreased to values between 7.0 and 7.5.

If calcium acetate complexes are formed and diffuse over the cell membrane, lower energy requirements for acetate transport should also be observed. In the present work, initial oxygen yields on acetate in experiments with calcium were in the range 0.16-0.18 $gO_2/gCOD_{HAc}$. In previous metabolic models for PHA production at pH 7, the minimum yield of oxygen per g of acetate was 0.25 $gO_2/gCOD_{HAc}$ (van Aalst-van Leeuwen et al., 1997). In this model, energy is required in the production of PHA from acetate in two steps: 1) to actively transport the acetate through the cell membrane and 2) to convert acetate to acetyl-CoA. At pH 7, each of these steps would require 1 ATP per mol of acetate. If passive transport through the cell membrane is included in this model, the minimum oxygen yield on acetate would be 0.13 and not 0.25 $gO_2/gCOD_{HAc}$, as observed in Figure 5.3. The initial oxygen yields observed in the current work suggest a potential contribution of passive acetate transport over the cell membrane.

Similarly, magnesium acetate complexes could be formed in $Mg(OH)_2$ experiments. However, even though $Mg^{2+}$ was present in equimolar concentration as those of $Ca^{2+}$ in $Ca(OH)_2$ experiments, it did not result to stimulate the effect of PHA accumulation with selective growth. In experiments with $Mg(OH)_2$, pH development and salinity were more similar to experiments with KOH rather than experiments with $Ca(OH)_2$. It remains unclear why or if magnesium would or should result in similar outcomes as calcium.

### 5.4.3. Simultaneous PHA production and selective biomass growth is promoted as consequence of the lower cellular energy requirements

As a consequence of the lower energy requirements, higher biomass and PHA production rates were observed. Biomass growth was identified to be the main driver to cause increased levels of PHA production. This growth response was selective towards the PHA-storing fraction of the biomass, as observed by increased degree of enrichment from the microscopy with staining and 16S rRNA gene analyses. At 48 h, 76-80% of the population was able to store PHA as compared to only 29-51% when KOH was used. Interestingly, the average PHA contents in just the PHA-storing biomass fraction of both reactors were estimated to be at similar levels, 0.58 and 0.63 gPHA/gVSS for KOH and $Ca(OH)_2$, respectively. In $Ca(OH)_2$ experiments, the sequencing data suggest that the biomass increase is at least partly due to the growth of *Ferribacterium* and *Zoogloea* species. The genus *Ferribacterium* is composed of strictly anaerobic chemo-organotrophs that oxidize organic acids with ferric iron, nitrate or fumarate as electron acceptor and it is associated with the production of PHB (Cummings et al., 1999). In WWTP Bath, iron is added to chemically removed phosphorus, and in the anoxic tank nitrate is present. These conditions likely explain the presence of this genus in the activated sludge from WWTP Bath, but they cannot explain why species of this genus (as identified) would have a competitive advantage in a fully aerated PHA accumulation reactor. For $Ca(OH)_2$ tests with four other activated sludge (see supplementary information), the growth response was associated with other well-known PHB producers from the genera *Pseudomonas*, *Zoogloea* and *Thauera* (Jiang et al., 2011b; Stouten et al., 2019; Queirós et al., 2015; Verlinden et al., 2007). Thus, certain species within the biomass seem to selectively benefit. Calcium may therefore not benefit all species of the PHA storing phenotype to the same extent. Further research should therefore focus on optimization of nutrients dosing and finding conditions that could potentially lead to the selective growth of superior PHA accumulators due to improved yields on substrate. Preliminary experiments with $Ca(OH)_2$ have indicated that a stricter nitrogen limitation resulted in similar PHA contents to KOH tests (data not shown) and this highlights the importance of biomass growth in the results that were obtained in the present work.

### 5.4.4. Implications for microbial community-based PHA production

Even if the mechanism(s) from which calcium promotes the selective growth of the PHA-storing biomass fraction remains to be elucidated, the outcomes have significant implications. In $Ca(OH)_2$ experiments a three-fold increase in PHA mass was obtained, compared to KOH, NaOH, $Mg(OH)_2$ experiments. This increase in PHA production allowed to reach PHA contents of up to 0.64 gPHA/gVSS, which are in line with the highest PHA contents reported to date with municipal activated sludge (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021a). These high PHA contents motivate to exploit municipal waste activated sludge as a generic resource for industrial scale PHA production (Figure 5.5).



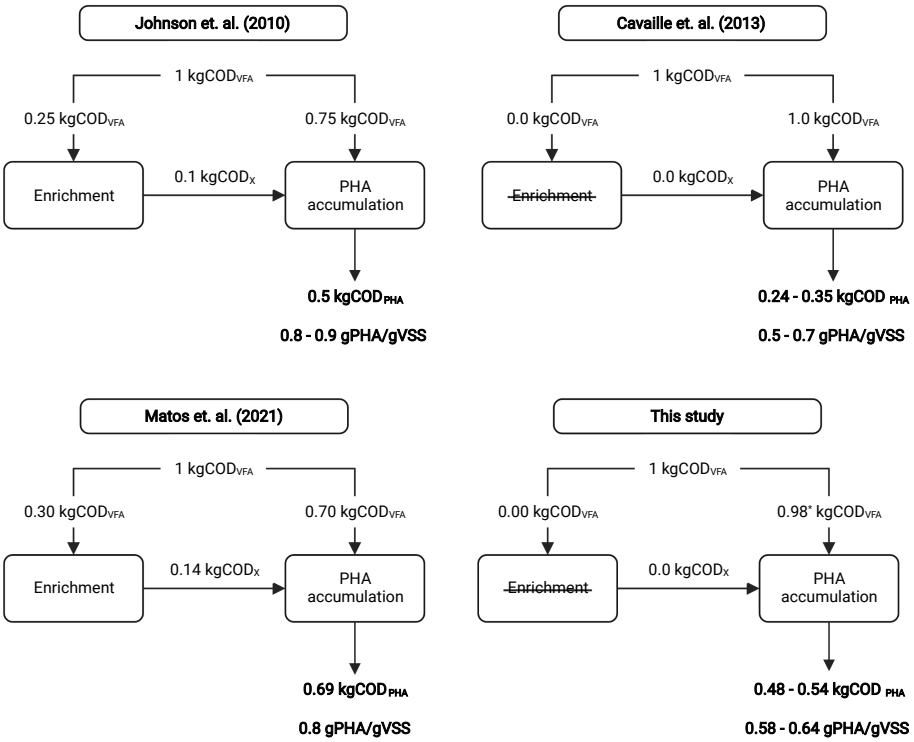


Figure 5.5. Comparison between the different PHA production routes: left) enrichment accumulation and right) direct accumulation. For the enrichment accumulation route, data is from Johnson et al. (2009a) and Matos et al. (2021). For the direct accumulation route, data is from Cavaillé et al. (2013) and this study. (*) In this study, 0.02 $kgCOD_{VFA}$ was used in the acclimation step. This figure was created by *Biorender*.

Additionally, the principles are suggestive for PHA production process strategies that can be applied generically for any activated sludge, as was also observed in this work. Differences in the maximum PHA content to be achieved will naturally be dependent on the fraction of PHA-storing biomass at the start. A higher fraction of PHA-storing biomass in the activated sludge would likely result in a higher final PHA content. Apart from this, the results suggest that specific species of PHA storing bacteria may benefit from the effects of lower yields on substrate due to presence of calcium. Therefore, even certain enrichment cultures could produce more polymer with less substrate. It would be of specific interest to explore the effect of calcium expanded to more highly enriched cultures, such as a *Plasticicumulans acidivorans* dominated culture.



In the present work, an excess of calcium was added to the feedstock in the form of $Ca(OH)_2$. However, in practical scenarios with waste streams, this approach is not feasible. Alternatively, calcium may already be present in the waste stream or otherwise calcium salts can be added to optimal concentrations directly to the mixed liquor. It is critical to determine the minimum criteria of soluble calcium concentration and pH that are necessary to efficiently drive the kind of simultaneous growth and accumulation response observed in this work. For instance, in the $Ca(OH)_2$ experiments performed in the current work, the concentration of calcium in the liquid did not exceeded 500 $mgCa^{2+}/L$, due to calcium carbonate precipitation. If it is found that a high calcium concentration is necessarily required to drive simultaneous growth and accumulation response, strategies to separate the precipitated calcium carbonate from the organic solids will need to be further developed. In this work, calcium carbonate was removed by acidification with HCl. Post accumulation acidification is known to be beneficial for the polymer stability for its preservation before downstream processing (Bengtsson et al., 2017b).

## 5.5. Conclusions

The presence of calcium resulted in PHA production with simultaneous selective growth of the PHA-storing biomass in waste activated sludge. As a result, higher PHA contents up to 0.64 gPHA/gVSS and consistently higher PHA yields on substrate, 0.49-0.55 $gCOD_{PHA}/gCOD_{HAc}$, were obtained using a full-scale waste ac-

tivated sludge and within a relatively short production cycle. This strategy opens a potential to apply these principles generically for any activated sludge, as long as there is already sufficient enrichment of the PHA-storing phenotype in the activated sludge to enable for short-term enrichment with concurrent PHA storage.

## Appendix

This appendix illustrates the documented functionalities of main community members found in calcium and potassium reactors. This appendix is inspired by the previous work of Gerben Stouten and co-workers (Stouten et al., 2019). and based on the Midas Field Guide database (Dueholm et al., 2022).



- *Ferribacterium*. Strictly anaerobic chemo-organotrophs that oxidize organic acids with ferric iron, nitrate or fumarate as electron acceptor and it is associated with the production of PHB (Cummings et al., 1999)
- *Zoogloea*. Zoogloea is a well known PHA-storing genus associated with the production of PHB and is often found in colder environments (Stouten et al., 2019).
- *Acinetobacter*. Acinetobacter is a strictly aerobic heterotrophic bacteria associated with the production of polyphosphate and PHA (Saunders et al., 2016).
- *Thauera*: Denitrifying bacteria typically found in activated sludge systems capable of PHA production (Thomsen et al., 2007).
- *Hydrogenophaga*: Chemoorganotrophic and facultative chemolithoautotrophic bacteria capable of PHA production from organic acids (Crognale et al., 2019).
- *Nitrospira*: Aerobic chemolithoautotrophic bacteria considered to be the most common nitrate oxidizing bacteria in wastewater treatment systems (Daims et al., 2001).
- *Flavobacterium*: Obligate aerobic bacteria, chemoorganictrophic typically

found in activated sludge systems and lately found in some PHA enrichment reactors (Wang et al., 2017).



To Chapter 4

## 5.6. Supplementary material

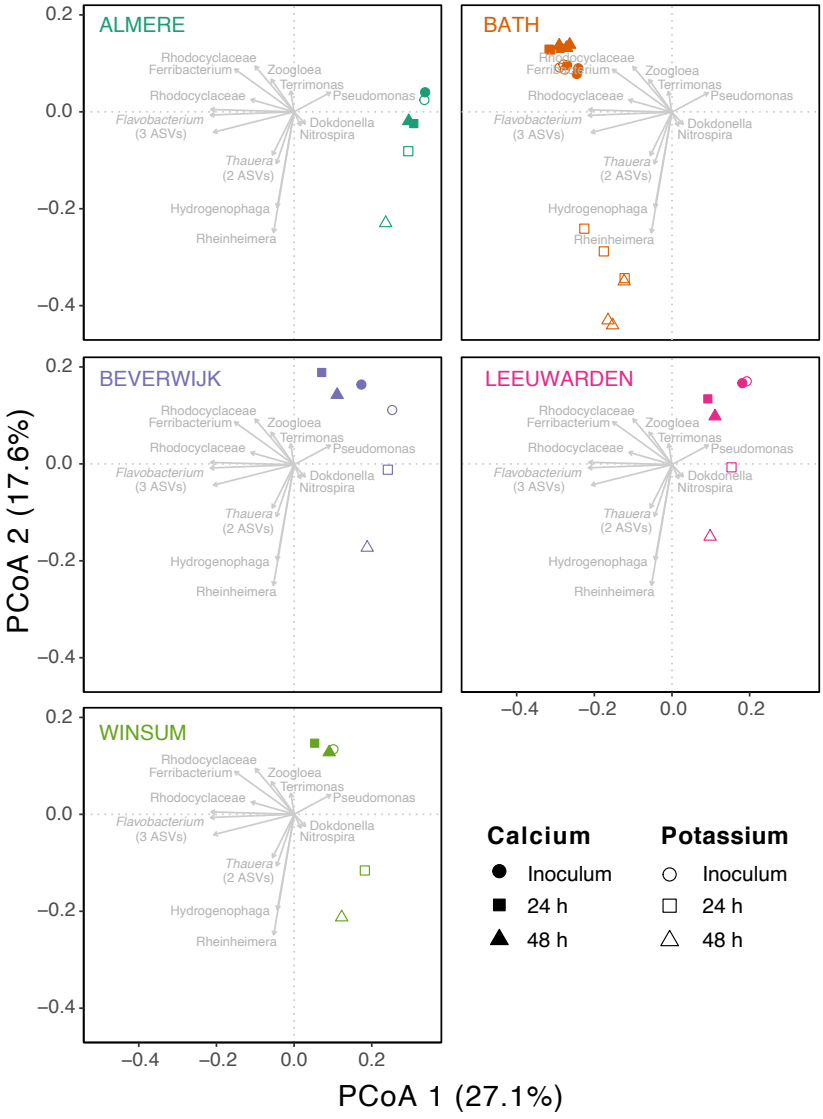


Figure 5.6. Principal coordinate analysis (PCoA) of the microbial communities across activated sludge types. Microbial communities of different wastewater treatment plants show different composition (Bray-Curtis dissimilarity, rarefied data) that aligns with their activated sludge compositions. The direction and strength (arrow length) of 15 most abundant genus across the dataset are shown.

Table 5.2. PHA contents and PHA mass after 48 hours accumulations with activated sludge from four different municipal WWTPs and with KOH and $Ca(OH)_2$ in the feedstock.

| | PHA content gPHA/gVSS | | PHA mass mgPHA | | |
|---|---|---|---|---|---|
| WWTP | KOH | $Ca(OH)_2$ | KOH | $Ca(OH)_2$ | Mass increase |
| Leeuwarden | 0.30 | 0.44 | 1190 | 2828 | x2 |
| Beverwijk | 0.42 | 0.50 | 2490 | 5560 | x2 |
| Almere | 0.18 | 0.34 | 746 | 2231 | x3 |
| Winsum | 0.23 | 0.44 | 715 | 2340 | x3 |

# 6

# Simultaneous nitrification and denitrification in microbial community-based PHA production

This chapter has been published as **Estévez-Alonso, Á.**, van Loosdrecht, M.C.M., Kleerebezem, R., Werker, A., 2021. *Simultaneous nitrification and denitrification in microbial community-based polyhydroxyalkanoate production.* Bioresource Technology, **337**, 125420.

## Abstract

*Microbial community based polyhydroxyalkanoate production has been demonstrated repeatedly at pilot scale. Ammonium, normally present in waste streams, might be oxidized by nitrifying bacteria resulting in additional aeration energy demand. The use of low dissolved oxygen (DO) concentrations allowed to reduce nitrifying rates by up to 70% compared to non-oxygen limiting conditions. At lower DO concentrations nitrate was used as alternative electron acceptor for PHA production and therefore, a constant PHA production rate could only be maintained if nitrate was sufficiently available. An optimum DO concentration (0.9 $mgO_2/L$) was found for which nitrification was mitigated but also exploited to supply requisite heterotrophic nitrate requirements that maintained maximum PHA production rates. PHA accumulations with such DO control was estimated to reduce oxygen demand by about 18%. This work contributes to establish fundamental insight towards viable industrial practice with the control and exploitation of nitrifying bacteria in microbial community-based PHA production.*

## 6.1. Introduction

Polyhydroxyalkanoates (PHA) are biodegradable polyesters that are naturally produced intracellularly by a wide range of species of microorganisms. PHA are energy and carbon storage polymers used by bacteria to cope with dynamic environments such as the alternating presence and absence of substrate (Van Loosdrecht et al., 1997). PHA have very interesting properties as materials for the polymer industry (Philip et al., 2007) and are commercially produced today using pure cultures and simple sugars as feedstocks (Li and Wilkins, 2020). However, in the last decade a progression of developments with microbial community-based PHA production has led to an alternative production approach with several successful pilot-scale installations (Kourmentza et al., 2017; Sabapathy et al., 2020; Estévez-Alonso et al., 2021a). The microbial community-based approach instead of pure cultures for PHA production allows for anticipated lowering of production costs and for utilization of a wide range of low value unrefined feedstocks, which are managed today as waste streams (Rodriguez Perez et al., 2018). Most of these PHA production pilot systems to date have used PHA-storing biomass produced explicitly with an enrichment of PHA-storing activity, or harvested directly as waste activated sludge from municipal/industrial wastewater treatment (Estévez-Alonso et al., 2021a). In both cases, high PHA contents have been reached and pilot-scale demonstration indicates the ability to engineer and maintain product quality and control even using fermented organic waste and/or municipal waste activated sludge as the volatile fatty acid (VFA) and biomass as input materials for the biopolymer production.



The use of fermented organic waste streams for microbial community-based PHA production introduces technical challenges due to the concomitant release of growth nutrients (mainly nitrogen and phosphorus) during the fermentation process (Capson-Tojo et al., 2016). The presence of growth nutrients in the feedstock plays a key role in the allocation of carbon towards PHA or biomass in PHA-storing bacteria (Johnson et al., 2010a; Valentino et al., 2015). Phosphorus levels may be readily reduced by chemical precipitation, but nitrogen is more difficult to be removed (Wilfert et al., 2015). Bioavailable nitrogen may promote active growth over storage in PHA-storing bacteria, but also allow for a wider range of micro-

bial growth activity, including so-called flanking populations of non PHA-storing bacteria (Valentino et al., 2015). An example of flanking population of non PHA-storing bacteria occurring when ammonium or nitrite are present in the process water is nitrification. Nitrification has been frequently observed at lab- and pilot-scale in microbial community-based PHA production when municipal waste activated sludge was used as PHA-storing biomass (Bengtsson et al., 2017a,b; Conca et al., 2020; Morgan-Sagastume et al., 2015) or when allylthiourea was not added to the feedstock (Third et al., 2003a; Fra-Vázquez et al., 2019; Morgan-Sagastume et al., 2020). Nitrifying bacteria are characterized by high oxygen consumption yields and low biomass production rates (Wiesmann, 1994), resulting in additional aeration energy demand. Alkalinity is also consumed and this introduces a loss of buffer capacity which may be required when acidic substrates are used within the process.

Several strategies are known to mitigate the growth of nitrifying microorganisms (Antoniou et al., 1990; Mulder et al., 2001; Pollice et al., 2002). Nonetheless, a challenge in a microbial community-based PHA production process is to control the extant activity of an established nitrifying community that is an inherent component of the PHA-storing biomass. In laboratory scale PHA production research, allylthiourea is used to eliminate nitrification activity, but it is unsuitable for industry scale application (Liu et al., 2018). High pH has also not been enough to limit the activity of nitrifying bacteria in a PHA production process even with highly functionalized biomass (Fra-Vázquez et al., 2019). However, low dissolved oxygen (DO) concentrations have shown promise (Third et al., 2003b; Reddy and Mohan, 2012; Wang et al., 2019). Based on batch activity tests with waste activated sludge, Wang et al. (2019) found that it should be possible to limit nitrification in a PHA accumulation process by lower DO concentrations. PHA-storing bacteria were also negatively affected by lower DO concentrations and simultaneous nitrification and denitrification (SND) was observed when both nitrifying and PHA-storing bacteria were active. These published results promoted an idea for a strategy of DO level control to mitigate flanking nitrifying bacteria, but also introduce the possibility to sustain PHA production productivity by promoting concurrent denitrification activity in the activated sludge flocs. For a likely relevant PHA-

storing biomass at industrial scale, the question, of the extent to which SND activity may be exploited during microbial community-based PHA production, has not yet been systematically explored.

The aim of this work was to evaluate the extent of positive influence simultaneous nitrification and denitrification could bring to an industrial PHA production process. For this purpose, short-term batch PHA-accumulation experiments over a selected range of constant DO concentrations were performed in order to quantify and compare the nitrification, denitrification and PHA production process rates in isolation and in combination. This work aimed to establish fundamental insight towards implementing a strategy of DO control and to critically assess the benefit that such control strategy could bring to industrial practice of microbial community-based PHA production. Nitrogen rich feedstocks and PHA-storing biomass that inherently have nitrifiers present are anticipated to become commonly available as starting resource materials in future value chains of PHA production (Bengtsson et al., 2017b).



## 6.2. Materials and methods

### 6.2.1. Experimental set-up

Stimulus-response experiments were performed in double-jacketed glass bioreactors (1 L working volume). Two reactors were operated in parallel and thermostated at 25 ± 0.1°C. Agitation was performed with two standard three-bladed turbines (R16 and R20, CAT Scientific, Germany) at a stirring rate of 150 rpm. pH was controlled between 7 and 7.5 by automatic titration with 1 M HCl (VWR Chemicals, The Netherlands). Airflow rate was controlled in the range between 0-2 L/min by a PID mass flow controller (FG-201CV-RGD-22-V-AA-000, Bronkhorst, The Netherlands) for selected set-point mixed liquor DO concentrations. DO and pH probes (COS81D and CPS11D, Endress & Hausser, The Netherlands), pH control pumps (Stepdos 10, KNF, The Netherlands) and mass flow controllers were connected and controlled by a 4-channel transmitter box (Liquiline CM444, Endress & Hausser, The Netherlands). The transmitter box also provided for data logging at a 10 s interval. DO and pH probes were calibrated before each experiment

according to the manufacturer's instructions.

### 6.2.2. Sludge source and feedstock

Waste activated sludge from the municipal wastewater treatment plant (WWTP) Bath (Rilland-Bath, The Netherlands) was used as the PHA-storing biomass for all experiments. WWTP Bath (470,000 PE) consists of a primary separation by screening and primary sedimentation followed by a modified Ludzack-Ettinger biological process (pre-denitrification and nitrification) treating a mix of regional municipal and industrial waste-water. This biomass was selected based on its well-established and consistent level of performance for PHA accumulation (Bengtsson et al., 2017b). Fresh gravity belt thickened waste activated sludge (57.8 ± 1.2 gTS/kg and 40.6 ± 1.4 gVS/kg (n=5)) was delivered batchwise by courier every two weeks in 20 L carboys and stored at 4°C pending experiments. Benchmark evaluations found no evidence to support that storage of samples at 4°C up to 10 days have any significant effect in both nitrifying and PHA-storing capacity of this activated sludge. In total, 5 distinct batches were used for the experiments performed.



The accumulation feedstock, with nutrients ratio 100:5:2.5 (COD:N:P by weight), was prepared with tap water and was as follows: 80 g/L acetic acid, 19 g/L $NH_4Cl$, 10.96 g/L $KH_2PO_4$ and 20 mg/L allylthiourea. Acetic acid and/or allylthiourea were included or omitted from the feedstock depending on the activity assay that was to be performed, as shown in Table 6.1. The pH of the feedstock was adjusted to pH 6 with KOH pellets.

### 6.2.3. Batch assays at selected dissolved oxygen concentrations

A total of 24 assays, divided into three different sets of experiments, were performed to evaluate PHA-storing and nitrifying bacteria activities in combination and in isolation, over a selected range of DO concentrations (Table 6.1). For each assay, a 30 g grab sample from the stored thickened activated sludge was diluted with tap water to nominally 1 gVSS/L, and 1 L was dispensed to the two parallel reactors set to 25 °C. Reactor mixed liquor was conditioned by aeration overnight at DO levels greater than 5 $mgO_2$/L to oxidize all the easily biodegradable COD, am-

Table 6.1. Overview of the batch assays performed at selected DO concentrations of 0.5, 1, 3 and 5 $mgO_2/L$. Batch HT(+)N(-) was to quantify the activity of PHA-storing bacteria in absence of nitrification. Batch HT(-)N(+) was to quantify nitrifying activity in absence of heterotrophic activity. Batch HT(+)N(+) was to quantify the activity of both PHA-storing and nitrifying bacteria simultaneously. ATU = allylthiourea.

| Batch | Acetate | Ammonium | Nitrate | ATU | Phosphate |
|---|---|---|---|---|---|
| HT(+)N(-) | Yes | Yes | Yes | Yes | Yes |
| HT(-)N(+) | No | Yes | Yes | No | Yes |
| HT(+)N(+) | Yes | Yes | Yes | No | Yes |

monium and nitrite present in the water and to establish a common baseline of endogenous microbial activity between tests. Because ammonium and nitrite were oxidized to nitrate during conditioning, all the experimental dose-response assays started with an initial background nitrate concentration of 9-13 $mgN\text{-}NO_3/L$. Subsequently, 5 mL of feedstock was added as a single pulse input, and DO concentration was then regulated to one of four selected set points within a ± 0.1 $mgO_2/L$ error margin: 0.5, 1, 3 or 5 $mgO_2/L$. Selected set-points were reached within 10 min and this time was used to determine maximum oxygen consumption rates. The three types of assay applied are explained in Table 6.1.



### 6.2.4. Analytical methods

During the batch assays, the process was monitored by online data logging (DO, temperature, pH and airflow rate) and from 45 mL hourly grab samples for water quality and solids analyses. Grab samples from the well-mixed vessel comprised three 15 mL aliquots: two aliquots were used for solids and liquid analyses, and the third was applied for biomass PHA content determination. Suspended solids were separated from the mixed liquor by centrifugation (3250 RCF and 4°C for 20 minutes). The supernatant after membrane filtration (0.45 $\mu$m pore size filters) was stored at -20°C pending analyses within 72 h. The harvested biomass pellet dry weight and volatile solids contents were estimated based on Standard Methods (105°C drying and 550°C ashing (Clesceri et al., 1999)), and these were referenced to the sample volume to estimate total and volatile suspended solids (TSS

and VSS), respectively. Soluble chemical oxygen demand (sCOD) was determined by Hach-Lange (Germany) LCK014 and LCK314 test kits. Acetic acid concentration was determined by ultra high pressure liquid chromatography (UHPLC) using a Dionex Ultimate 3000RS system equipped with a Phenomenex Rezex Organic Acid $H^+$ column (300x7.8 mm) and a Dionex Ultimate 3000 RS UV detector (210 nm) with 2.5 mM sulfuric acid mobile phase at 0.5 mL/min and 80°C. Ammonium, nitrite, nitrate and phosphate concentrations were determined by ion chromatography or IC (Metrohm Compact IC Flex 930, Metrohm, Switzerland) with conductivity detector. For ammonium determination, a pre-column (Metrohm Metrosep RP 2 Guard/3.6) and a column (Metrohm Metrosep C4-150/4.0 mm) and 2.5 mM nitric acid mobile phase at 0.9 mL/min and room temperature were applied. For nitrite, nitrate and phosphate determination, two pre-columns (Metrohm Metrosep RP 2 Guard/3.6 and Metrohm Metrosep A Supp 4/5 Guard) and a column (Metrohm Metrosep A Supp 5, 150/4.0 mm) with 3.2 mM sodium carbonate and 1 mM sodium bicarbonate + 1% acetone solution mobile phase at 0.7 mL/mL and room temperature were used. Additionally a chemical suppressor was applied (0.2 M phosphoric acid + 1% acetone at 0.1 mL/mL).



The aliquot for PHA determination was directly acidified to pH 2 with 20 $\mu$L of $H_2SO_4$. After thorough mixing (5 min) suspended solids were collected (3250 RCF and 4°C for 20 min). The biomass pellet was retained and dried at 105°C. Dried pellets were ground and analyzed by thermogravimetric analysis or TGA (TGA 2, Mettler Toledo, The Netherlands). A 5 mg of ground sub-sample was introduced to the furnace at 80°C, and heated to 105°C (10 °C /min) under nitrogen atmosphere. After drying at 105°C for 10 min, the sample was heated to 550°C (10 °C/min), under nitrogen atmosphere. At 550°C, the atmosphere was switched to air and temperature was held for 30 min. The weight loss trends were used to determine the sample residual moisture content and the biomass PHA fraction with respect to the biomass dried total and volatile solids as previously described (Chan et al., 2017). The PHA content was expressed as fraction of the biomass volatile solids (gPHA/gVSS). The biomass fraction was estimated as the total biomass as VSS minus PHA content. Biomass was chemically represented as $CH_{1.8}O_{0.5}N_{0.2}$ (Roels, 1980).

### 6.2.5. Mass balance and process modeling

For each batch assay, biomass specific rates and yields on substrate were estimated based on mass balance principles and a process model. Both on and offline monitoring data were applied. This model was adapted from Tamis et al. (2014b) and was used to estimate the biomass specific rates for the heterotrophic fraction of the biomass: acetic acid uptake rate ($q_{HAc}$), PHA yield on substrate ($Y_{PHA,HAc}$), PHA degradation rate ($k_{PHA}$) and heterotrophic nitrate uptake rate ($q_{NO3}$). Nitrifying biomass specific rates, ammonium uptake rate ($q_{AOB}$) and nitrite uptake rate ($q_{NOB}$) were incorporated to the model by means of a modified version of the activated sludge models (Iacopozzi et al., 2007). Bacterial growth (heterotrophic and autotrophic) was not included in the process model and microbial rates were calculated on a total VSS basis. All model kinetic equations are shown in Table 6.2.

The model was applied to estimate the parameter set that minimized the sum

Table 6.2. Model kinetics used for the data fitting.

| | | |
|---|---|---|
| **Heterotrophic bacteria** | | |
| HAc uptake | $q_{HAc} = q_{HAc}^{max}\left(\frac{C_{HAc}}{k_{HAc}+C_{HAc}}\right)$ | - |
| PHA production | $q_{PHA}^{+} = q_{HAc}\cdot Y_{PHA,HAc}$ | if $C_{HAc} > 0$ |
| PHA degradation | $q_{PHA}^{-} = k\cdot\left(\frac{C_{X0}}{C_X}\right)^{1/3}\cdot f_{PHA}^{2/3}$ | if $C_{HAc} = 0$ |
| $NO_3^-$ uptake | $q_{NO_3} = q_{NO_3}^{max}\left(\frac{C_{NO_3}}{k_{NO_3}+C_{NO_3}}\right)$ | if $C_{HAc} > 0$ |
| **Nitrifying bacteria** | | |
| $NH_4^+$ uptake | $q_{AOB} = q_{AOB}^{max}\left(\frac{C_{NH_4^+}}{k_{NH_4^+}+C_{NH_4^+}}\right)$ | - |
| $NO_2^-$ uptake | $q_{NOB} = q_{NOB}^{max}\left(\frac{C_{NO_2^-}}{k_{NO_2^-}+C_{NO_2^-}}\right)$ | - |

of the squared relative error with respect to measured variables for each respective individual experiment. Subsequently, a second kinetic model estimated the overall average response of the biomass with respect to maximum specific substrate uptake rates ($q_{\mathrm{HAc}}^{max}$) and apparent affinity constant for oxygen ($k_{DO}$) as function of DO, as defined in Equations 6.1 and 6.2. It was assumed that oxygen was the only limiting substrate and denitrification would only begin to take place when oxygen became sufficiently limiting, based on an affinity constant $k_{DO}$. The $q_{\mathrm{HAc}}^{max}$ was assumed to be independent of electron acceptor and therefore, $k_{DO}$ provided an estimate of the balance of aerobic/anoxic contributions to heterotrophic substrate uptake rates. The nitrate yield ($Y_{\mathrm{NO_3,HAc}}$) was assumed to be constant and was 0.2 gN-$NO_3$/gCOD (Beun et al., 2000b). These equations were implemented in Microsoft Excel and the function SOLVER (Generalized Reduced Gradient Nonlinear algorithm) was used to minimize the sum of squared relative error between the ensemble of fitted data from all the batch experiments.



$$q_{\mathrm{HAc}} = q_{\mathrm{HAc}}^{aerobic} + q_{\mathrm{HAc}}^{anoxic} = q_{\mathrm{HAc}}^{max}\left(\frac{DO}{k_{DO}+DO}\right) + q_{\mathrm{HAc}}^{max}\left(\frac{k_{DO}}{k_{DO}+DO}\right) \tag{6.1}$$

$$q_{\mathrm{NO_3}} = q_{\mathrm{NO_3}}^{anoxic} \cdot Y_{\mathrm{NO_3,HAc}} \tag{6.2}$$

## 6.3. Results and discussion

A total of 24 assays were performed with the activated sludge to evaluate the PHA-storing and nitrifying activity, in combination and in isolation, over a selected range of DO concentrations (Table 6.1). One of the assays at 5 mg$O_2$/L with the measured data and its model characterization is provided, as a typical example, in Figure 6.1. All the other experiments followed similar systematic trends and the data set can be found in Mendeley Data (https://data.mendeley.com/datasets/gnnkdpg3rp/1).

### 6.3.1. PHA storage in absence of nitrification

Four batch tests without nitrification were performed to determine reference aerobic and anoxic acetate uptake rate, PHA production rate and the yield of PHA on acetate as function of DO concentration. The absence of nitrification allowed to study the heterotrophic activity only. A pulse containing acetate, ammonium and ATU was added to the mixed liquor that had the above mentioned background $NO_3$ concentration. An overview of results for the four assays is provided in Figure 6.2 and Table 6.3.

The aerobic acetate uptake rate was not measurably affected by low DO concentrations and was estimated to be 154.7 mgCOD/(gVSS·h). Low denitrification rates were observed at 0.5 $mgO_2/L$, $q_{NO_3}$ = 0.34 $mgN\text{-}NO_3/(gVSS{\cdot}h)$, which represented less than 1% of the total respiration rate, based on electron equivalents. The low denitrification rates at low DO concentration indicate that the tests were successfully performed under fully aerobic conditions. Besides, a negligible apparent $k_{DO}^{HT}$, 0.01 $mgO_2/L$, was identified. A low apparent $k_{DO}^{HT}$ indicates a high affinity for oxygen which resulted in the ability to maintain maximum uptake rates even at low DO concentrations. Because heterotrophic activity could be maintain at maximum rates at low DO concentrations, the yields of PHA on acetate were



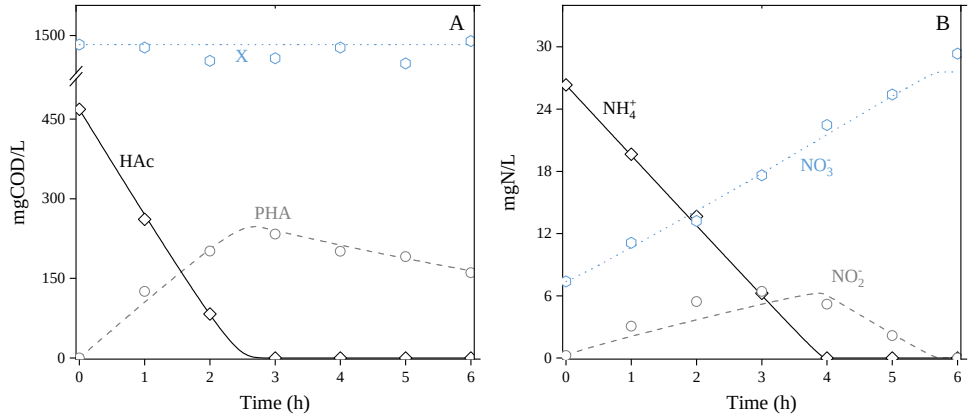


Figure 6.1. Detailed characterization of a representative test at 5 $mgO_2/L$ with active PHA production and nitrification including model fitting and measurements of A) acetate, PHA and biomass (X) and B) ammonium, nitrite and nitrate.

also not affected by the lower DO concentrations and were in the range 0.5-0.6 $gCOD_{PHA}/gCOD_{HAc}$. The PHA yields on substrate obtained in this work were similar, and close to the theoretical maximum yields, to those obtained previously under fully aerobic conditions, 0.5-0.7 $gCOD_{PHA}/gCOD_{HAc}$ (Morgan-Sagastume et al., 2015; Bengtsson et al., 2017b,a; Wang et al., 2019; Conca et al., 2020).

The average ammonium uptake rate was 1.33 $mgN\text{-}NH_4/(gVSS{\cdot}h)$ and was assumed to be exclusively due to assimilation by the heterotrophic bacteria, as ATU was present in the feedstock to block nitrification. The average ammonium uptake rate was used in the modelling as ammonium uptake for heterotrophic growth in tests HT(+)N(+).

### 6.3.2. Nitrification rates

Eight batch assays were used to characterize the ammonium and nitrite uptake rate as function of DO concentration, when both ammonium oxidation (AOB) and nitrite oxidation bacteria (NOB) were dominantly active due to absence of added organic substrate. A pulse of ammonium was added to the mixed liquor also containing a background $NO_3$ concentration. An overview of the eight assays is shown in Figure 6.2 and Table 6.3.

Both ammonium and nitrite uptake rates decreased at lower DO concentrations: a 49% lower $q_{AOB}$ was observed at 0.5 $mgO_2/L$ compared to the model maximum $q_{AOB}^{max}$. Consequently an apparent $k_{DO}^{AOB}$ of 0.49 $mgO_2/L$ was estimated for this activated sludge AOBs. Similar results were obtained for NOBs at 0.5 $mgO_2/L$, with a 50% lower $q_{NOB}$ with respect to the maximum $q_{NOB}^{max}$ and an apparent $k_{DO}^{NOB}$ of 0.50 $mgO_2/L$. The higher apparent $k_{DO}$ obtained for nitrifying bacteria compared to heterotrophic bacteria indicates that low DO concentrations have a more detrimental effect in nitrifying than in heterotrophic bacteria. At lowered DO concentrations, therefore, nitrifying bacteria will be more progressively limited compared to the heterotrophic bacteria. The apparent $k_{DO}$ includes not only biological but also physical mass transfer rate dependent phenomena, such as the size of the cell clusters and/or the size of the floc, which also depends on the mixing intensity (Picioreanu et al., 2016; Pérez et al., 2005; Manser et al., 2005; Chu et al., 2003). Nonetheless, the apparent $k_{DO}$ and rates obtained for nitrifying bacteria are

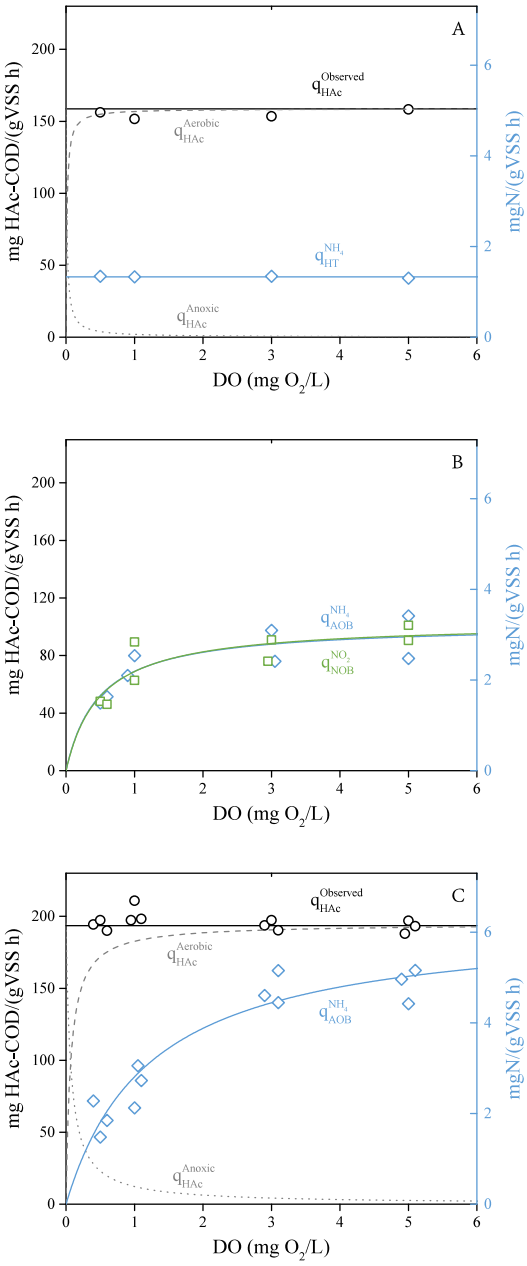


Figure 6.2. Aerobic and anoxic acetic acid, ammonium and nitrite uptake rates as function of DO concentration in tests where only heterotrophic bacteria (A), only nitrifying bacteria (B) and both nitrifying and heterotrophic bacteria (C) were active. The symbols represent the microbial rates derived from each batch assay and the lines the model results from Equation 6.1.

Table 6.3. Model derived variables for heterotrophic and nitrifying bacteria in assays tests where only heterotrophic bacteria (HT(+)N(-)), only nitrifying bacteria (HT(-)N(+)) and both nitrifying and heterotrophic bacteria (HT(+)N(+)) were active.

| | Heterotrophic bacteria | | Nitrifying bacteria | | |
|---|---|---|---|---|---|
| | HAc | | AOB | NOB | |
| **Batch HT(+)N(-)** | | | | | |
| $q^{max}$ | 154.7 | mgCOD/(gVSS·h) | n.d. | | mgN/(gVSS·h) |
| $k_{DO}^{App}$ | 0.01 | $mgO_2/L$ | n.d. | | $mgO_2/L$ |
| $Y_{PHA,HAc}$ | 0.58 ± 0.05 | mgCOD/mgCOD | | | |
| **Batch HT(+)N(+)** | | | | | |
| $q^{max}$ | 194.9 | mgCOD/(gVSS·h) | 6.26 | 4.42 | mgN/(gVSS·h) |
| $k_{DO}^{App}$ | 0.07 | $mgO_2/L$ | 1.23 | 0.68 | $mgO_2/L$ |
| $Y_{PHA,HAc}$ | 0.57 ± 0.04 | mgCOD/mgCOD | | | |
| **Batch HT(-)N(+)** | | | | | |
| $q^{max}$ | n.d | mgCOD/(gVSS·h) | 3.23 | 3.27 | mgN/(gVSS·h) |
| $k_{DO}^{App}$ | n.d | $mgO_2/L$ | 0.48 | 0.50 | $mgO_2/L$ |
| $Y_{PHA,HAc}$ | n.d. | mgCOD/mgCOD | | | |

in line with those previously reported for similar reactor conditions (Wang et al., 2019; Picioreanu et al., 2016; Arnaldos et al., 2015). As a result, the use of low DO concentrations should allow to run a PHA production process with predominant heterotrophic and lower nitrifying activity, which was further explored in Section 6.3.3.

### 6.3.3. Simultaneous PHA storage and nitrogen conversion

Twelve batch assays were used to characterize the heterotrophic aerobic and anoxic acetate uptake rates, PHA production rate and the yields of PHA on acetate in combination with the autotrophic AOBs ammonium uptake rate and the NOB nitrite uptake rate as function of DO concentration. A pulse of feedstock containing acetate and ammonium (with no ATU) was added to start these assays, again

with a background starting $NO_3$ concentration. An overview of the twelve assays is given in Figure 6.2 and Table 6.3.

The acetate uptake rate remained constant and seemingly independent of DO level. However, the maximum acetate uptake rate was 26% higher compared to the acetate only experiments: 194.9 compared to 154.7 $mgCOD_{HAc}/(gVSS\ h)$. The higher heterotrophic rates may be attributed to a naturally expected degree of batch-to-batch variability between the 5 activated sludge batches from a full-scale process and delivered over a period of 40 days from the WWTP. At lower DO concentrations, higher anoxic $q_{HAc}$ was estimated: 23.0 $mgCOD_{HAc}/(gVSS\ h)$ at 0.5 $mgO_2/L$ compared to 2.6 $mgCOD_{HAc}/(gVSS\ h)$ at 5 $mgO_2/L$. Consequently, the aerobic $q_{HAc}$ decreased at lowered DO concentrations, being 12% lower at 0.5 $mgO_2/L$ compared to the derived maximum $q_{HAc}^{max}$. This suggests that the use of nitrate as alternative electron acceptor allowed the PHA-storing bacteria to maintain a similar level of activity as observed under non-oxygen limiting conditions. Simultaneous nitrification and denitrification is often explained as a consequence of mass transfer limitations due to diffusion gradients, which leads to oxygen limitation within the floc/granule (Pochana and Keller, 1999). In those zones within the floc where oxygen is depleted, facultative aerobic bacteria can switch electron acceptor from oxygen to nitrate. The waste activated sludge used in this work was obtained from Bath WWTP, which is composed of a pre-denitrification (anoxic) and nitrification (aerobic) steps. It is expected that the Bath sludge is enriched in facultative aerobic bacteria that are able to switch electron acceptor from oxygen to nitrate, depending on the environmental conditions e.g. oxygen limitation within the floc. Oxygen limitation within the floc/granule is likely due to the presence of active nitrifying bacteria competing for the available oxygen, as no anoxic PHA production was observed in those tests where only heterotrophic bacteria were active. The presence of active nitrifying bacteria altered the diffusion of oxygen within the floc, reducing the penetration depth and the oxygen availability for aerobic PHA production in the floc. As introduced in Section 6.3.2, the apparent $k_{DO}$ includes not only biological but also physical mass transfer rate phenomena, and therefore, a higher apparent $k_{DO}^{HT}$ compared to the baseline experiments without active nitrification should be observed. The observed apparent $k_{DO}^{HT}$ was 0.07 $mgO_2/L$, almost

one order of magnitude higher than the baseline experiments without active nitrification, 0.01 $mgO_2/L$.

#### 6.3.3.1. PHA storage

Lowered DO concentrations did not influence the PHA yield on acetate, that were in the same range as in tests where only heterotrophic bacteria were active, 0.5-0.6 $gCOD_{PHA}/gCOD_{HAc}$. In this case, and as a result of mass transfer limitation within the floc, up to 15% of the obtained PHA has been produced under anoxic conditions. PHA yields on substrate under anoxic conditions are reported to be 40-60% lower than under aerobic conditions (Kuba et al., 1996; Beun et al., 2000a). However, an anticipated decrease in the overall PHA yield on substrate due to the anoxic PHA accumulation could not be resolved in this work, at least within the frame of the replicated experiments and the model used to fit these data. If the aerobic PHA yield on substrate would be 0.6 $gCOD_{PHA}/gCOD_{HAc}$ and the anoxic PHA yield on substrate would be 50% lower, 0.3 $gCOD_{PHA}/gCOD_{HAc}$, the expected



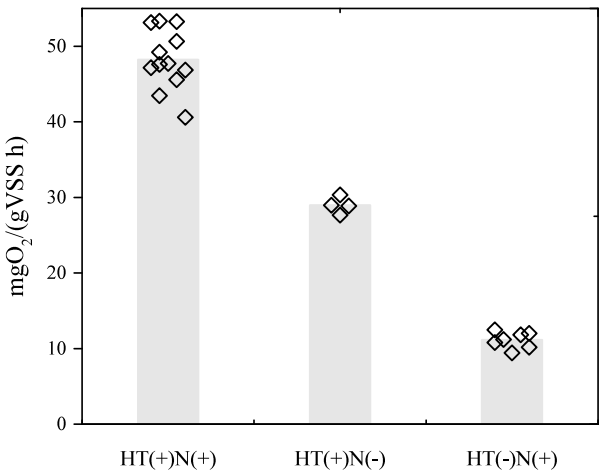


Figure 6.3. Maximum oxygen uptake rates in tests where only heterotrophic bacteria (HT(+)N(-)), only nitrifying bacteria (HT(-)N(+)) and both nitrifying and heterotrophic bacteria (HT(+)N(+)) were active. The symbols represent the microbial rates derived from each batch assay and the bars give the average values.

observed yield, when 15% of the PHA is produced anoxically, would be expected to have been lowered by 7% to 0.56 $gCOD_{PHA}/gCOD_{HAc}$.

#### 6.3.3.2. Nitrogen conversion

The maximum AOB uptake rate was higher (almost double) than in tests where only nitrifying bacteria were active: 6.3 mgN-$NH_4$/(gVSS·h) compared to 3.2 mgN-$NH_4$/(gVSS·h). The higher nitrifying uptake rates may not be explained only by a variation in the sludge samples. The observed higher rates are understood to have been promoted both by variations in airflows between experiments and a development of inorganic carbon limitation in tests where only nitrifying bacteria were active. During the conditioning of the biomass (overnight aeration) the dissolved inorganic carbon has been observed to become depleted due to gas stripping and nitrification. Sufficient loss of inorganic carbon could have led to conditions of carbon limitation for autotrophic microbial activity in tests where only nitrifying bacteria were active. In tests, with both nitrification and heterotrophic consumption on acetate, carbon dioxide is supplied by heterotrophic activity even if alkalinity is similarly depleted initially.

In line with tests HT(-)N(+) and with the previous work of Wang et al. (2019) with the same activated sludge, nitrification rates were negatively affected by the lower DO concentrations, the aerobic $q_{AOB}$ at 0.5 $mgO_2/L$ was 70% lower than the derived maximum $q_{AOB}^{max}$. Not only the presence of nitrifying bacteria affected the PHA-storing bacteria, as explained in Section 6.3.3.1, but also the PHA-storing bacteria had an effect on the nitrifying bacteria. This effect can be observed in the higher apparent $k_{DO}^{AOB}$. $k_{DO}^{AOB}$ in tests where both nitrifying and heterotrophic bacteria were active was 1.23 $mgO_2/L$, and two times higher than the one obtained in tests where only nitrification was active, 0.48 $mgO_2/L$ (Table 6.3). From these experiments, the maximum nitrifying oxygen consumption rate was also derived and would represent 23-27% of the total oxygen demand for a process under assumed steady state conditions, as shown in Figure 6.3. Nonetheless, if the nitrifying bacteria rates were indeed underestimated in tests where only nitrifying bacteria were active due to inorganic carbon limitation, then these predicted outcomes are conservative, and the nitrifying oxygen consumption rates could have represented up

to 40% for the total oxygen demand of the process.

### 6.3.4. Implications for microbial community-based PHA production

Microbial community-based PHA production research and development at laboratory and pilot-scales are usually run with an objective for fully aerobic conditions with fixed airflow rate and without a control or consideration for influence of autotrophic oxygen demand (Kourmentza et al., 2017; Rodriguez Perez et al., 2018; Sabapathy et al., 2020). This work introduces the possibility to operate the PHA accumulation process at fixed low DO concentrations and to consequently utilize a wide variety of feedstocks, ranging from both low to high ammonium concentrations. The ability to leverage the activity of nitrifiers relies on potential for facultative PHA-storing microbial activity. While running a PHA accumulation at low DO concentrations part of the PHA will be stored using nitrate as electron acceptor. Therefore, an optimum control point for DO for nitrifying activated sludge is achieved when the autotrophic nitrate production balances the heterotrophic nitrate requirements. The DO optimum level and respective denitrification rates



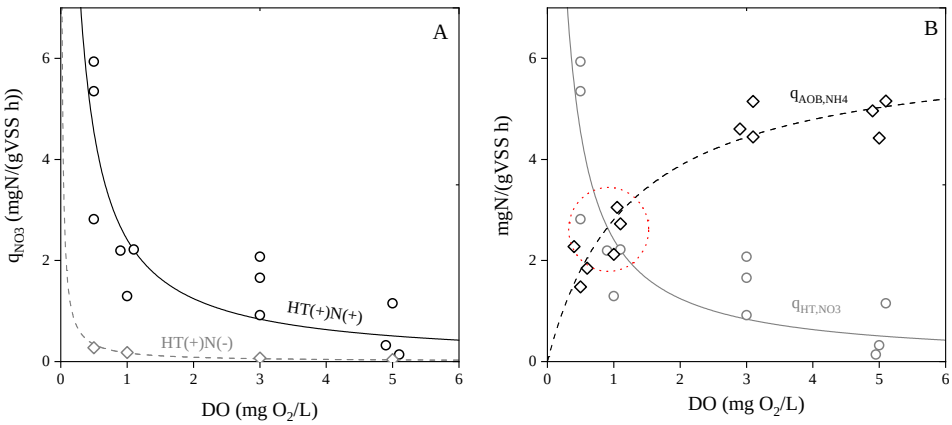


Figure 6.4. Heterotrophic nitrate consumption rates as function of DO concentration in tests where only heterotrophic bacteria (HT(+)N(-)) and both nitrifying and heterotrophic bacteria (HT(+)N(+)) were active (A) and heterotrophic nitrate and autotrophic ammonium consumption rates as function of DO concentration in tests where both nitrifying and heterotrophic bacteria were active (HT(+)N(+)) (B). The symbols represent the microbial rates derived from each batch assay and the lines the model results.

are expected to be tunable for biomass with different origin and enrichment strategy, as process specific conditions will influence floc size and mass transfer coefficients (Arnaldos et al., 2015). However, the fundamental principles are expected to be valid and generically applicable. For the biomass used in this work, the optimum point was found to be around 0.9 $mgO_2/L$ (Figure 6.4). Running a PHA accumulation with DO control level close the optimum allows to reduce the respiration demands of the process, conservatively, by 17 to 19% or to increase the reactor biomass loading (volumetric productivity) given the same vessel and aeration capacity. These outcomes are expected to influence practical outcomes of capital (CAPEX) and operating expenses (OPEX). Anoxic PHA production will also allow to lower the final nitrogen concentrations in the process effluent. Denitrification rates observed at 0.9 $mgO_2/L$ can allow to remove nitrogen from the liquid phase at removal rates of 2.7 mgN/(gVSS h). For a standard PHA accumulation run with waste activated sludge as biomass source and a duration of 16 h, 43 mgN/gVSS could be removed for this biomass. Nitrate is expected to be reduced to dinitrogen gas, but the fate of nitrogen requires validation as part of the ongoing research and development. If the denitrification process is not complete, nitrous oxide ($N_2O$) can be produced, which is a known greenhouse gas (Kampschreur et al., 2009). Most of the $N_2O$ emissions in wastewater treatment plants are associated with low DO concentrations in the nitrification tank. These conditions are very similar to those tested in this work, therefore further evaluation of this control strategy with respect to nitrogen fate with $N_2O$ and/or its control in PHA production at low DO concentration is recommended.



## 6.4. Conclusions

This study establishes methods for microbial community-based PHA production with nitrogen rich feedstocks and PHA-storing biomass containing nitrifying bacteria. Simultaneous nitrification and denitrification can be used to optimize polymer production, process aeration requirements and productivity. In this way, the aeration demand of the process can be reduced, conservatively, by 17 to 19% while reducing the effluent nitrogen discharge levels. The results of this work show that substrate sources containing ammonium can efficiently be used for microbial

community-based PHA production, widening the generic potential for substrate availability for PHA production from waste sources.



*To Chapter 3*

# 7

# Outlook

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This thesis together with previous research developments supports that it is possible to directly use municipal waste activated sludge as the microbial biomass input for industrial scale PHA production. Industrial processes must work to maximize yields of product outputs and minimize sources of process variability. However, the maximum biomass PHA content for municipal waste activated sludge are inherently variable between different municipal WWTPs, as observed in Chapters 3 and 4. The levels are also lower than those obtained when enrichment cultures are used for mixed culture PHA production (Estévez-Alonso et al., 2021b). The aim of this thesis was to evaluate principles that could be applied to the production of PHA by municipal activated sludge towards engineering the most optimal and robust possible outcomes. This research and development effort was divided into two parts. The first part was focused on defining and understanding causes for historically observed variability in PHA accumulation process performance with respect to biomass PHA contents and yields. The second part was then directed towards strategies that could help to mitigate production variability and maximize biomass PHA content and yields, while minimizing the activity of the non PHA-storing fraction in the biomass. Therefore, the outlook to integrate polymer production as a means for resource recovery with municipal wastewater treatment is positive. Steps have been made to advance the technological feasibility and improve the chances to reach this goal in practice. The work opens a door with new tools, insights and methods to help accelerate these developments and ambitions further. The main conclusions from the thesis work are given followed by recommendations for next steps and outlook.



## 7.1. Conclusions

The principal research and development conclusions are as follows:

- PHA molecular weights on average of 1500 kD and up to 2000 kDa can be obtained when municipal waste activated sludge is directly used for PHA production.
- The degree of enrichment for PHA-storing biomass in municipal activated sludge determines the maximum PHA content that can be achieved.

- Different municipal WWTPs with different operating conditions will result in different inherent degrees of enrichment for PHA-storing biomass.
- The maximum possible PHA content in the PHA-storing biomass fraction in municipal waste activated sludge is expected to be consistently equal to about 0.6 gPHA/gVSS.
- Selective biomass growth of the PHA-storing biomass fraction is beneficial for PHA production by municipal waste activated sludge.
- Calcium promotes conditions that favour selective growth of the PHA-storing biomass on acetate.
- Low dissolved oxygen concentrations during PHA accumulation (circa 1 $mgO_2/L$) can reduce oxygen demand from nitrifying microorganisms in waste activated sludge without decreasing PHA production rates.

## 7.2. Recommendations for future research



### 7.2.1. What drives selection for PHA-storing bacteria in municipal WWTPs?

In Chapter 4 it was observed that the PHA content in the PHA-storing biomass fraction present in municipal activated sludge was consistently around 0.6 gPHA/gVSS. Despite this high PHA content in the PHA-storing biomass fraction, the average biomass PHA contents were lower and not higher than 0.42 gPHA/gVSS. This difference was found to be due to differences in the fraction of PHA-storing biomass between WWTPs. From six different WWTPs the degree of enrichment ranged ranged from 0.16 to 0.52 v/v. This outcome raises two questions:

1. why the fraction of PHA-storing biomass in the municipal activated sludge remains below 0.5 v/v?
2. why bacteria present in municipal activated sludge can accumulate only around 0.6 gPHA/gVSS?.

PHA production and consumption is widely spread among bacteria and many aerobic and anoxic heterotrophic bacteria are able to accumulate and degrade PHAs (Pedrós-Alió et al., 1990; Gasser et al., 2009; Viljakainen and Hug, 2021). In municipal WWTPs, conditions are highly dynamic and one may think that most of the bacteria present in the activated sludge should be able to accumulate PHA. Microscopic observations show that only a fraction of the bacteria are storing significant amounts of PHA. Some suggestions were given in Chapter 4 on how to increase the degree of enrichment in municipal WWTPs. One aspect could be that a fraction of the bacteria is simply non-active. Microscopic quantification as used in Chapter 4 did not allow to distinguish between active and non-active bacteria. In a parallel research efforts, the percentage of non active biomass in activated sludge from one of the WWTP was estimated to be between 20 to 50% of the total biomass fraction. This suggests that, in fact, a large fraction of the non PHA-storing biomass fraction was composed of non-active biomass. Therefore, most of the active biomass was actively involved in PHA production for this WWTP. This fact cannot be extrapolated to municipal WWTPs in general. Some of the WWTPs evaluated exhibited a very low degrees of enrichment. In general, other factors related to the influent wastewater quality as well as the WWTP bioprocess configuration with its conditions of operation can be the cause higher or lower relative levels of activity and higher or lower degrees of enrichment. Principles and conditions in municipal WWTP that can promote maximal levels of the PHA storing phenotype in the active fraction need to be further understood and then exploited. The microscopy quantification methods from Chapter 4 can be further applied in longer term studies with systematic changes in operation for municipal wastewater treatment at lab- or even full-scale where cause and effect hypothesis can be explicitly tested.



It is also a puzzle to know why certain species of bacteria are able to accumulate PHA up to 0.9 gPHA/gVSS while others only accumulate 0.6 gPHA/gVSS (Kourmentza et al., 2017). The cell cytoplasm is crowded with all kinds of organelles and, so, available intracellular space is expected to be limited (Fulton, 1982; Spitzer and Poolman, 2013). Bacteria able to reach PHA contents up to 0.9 gPHA/gVSS are observed to be large and with observed capacity to increase cell size significantly

(Johnson et al., 2009a; Jiang et al., 2011c; Marang et al., 2013). Parallel research suggested that activated sludge samples contained both rigid and more flexible phenotypes of PHA storing bacteria (Pei et al., 2022a). The average PHA content of the PHA accumulating microorganisms was about 0.6 gPHA/gVSS, but the distribution can be broad or even bimodal. The actual PHA storage distribution in mixed cultures requires further investigation. Additionally, is there an ecological role or mechanism (advantage or disadvantage) for a given microorganism to accumulate up to a maximum of 0.9 gPHA/gVSS compared to 0.6 gPHA/gVSS in a natural environment beyond an extreme feast-famine cycle?.

### 7.2.2. Calcium and selective growth during accumulation

In Chapter 5 it was shown that the presence of calcium favors PHA production and promotes the selective growth of PHA-storing biomass. The experiments with calcium addition in this thesis were not optimized. The amount of calcium added was probably excessive, but also the amount of nutrients in the feedstock were based on previous experiences where calcium was not added. In the experiments with calcium addition, the aim should be to maximize the growth of the PHA-storing biomass, as more PHA can be produced. For this, the C/N ratio is expected to play a key role and should be further optimized.



The specific reason or mechanism of why calcium benefits simultaneous selected biomass growth with PHA production could not be determined. Further research should seek to develop an experimental design to confirm if the mechanisms supporting these beneficial effects do or do not require calcium carbonate precipitation. If calcium carbonate precipitation is not an element, it should be possible to identify conditions to robustly promote selective biomass growth and PHA accumulation with and without the presence of calcium. Respirometry analysis with different background calcium concentrations can be easily performed to estimate yields on oxygen. Based on the analysis provided in Chapter 5, a higher background calcium concentration is expected to result in a lower oxygen yield on substrate. If these conditions are found, the effect of other ions can be easily tested. In such a system, it will also be possible to study the physiological response of the biomass to calcium and validate the running hypothesis about the

role of calcium in bacteria, as proposed in Chapter 5. PHA production may also involve more complex volatile fatty acid mixtures as substrates. Can these principles be applied with other feedstocks that will be used in practice like fermented primary sludge? If the cause is due to the interpreted effect of calcium supporting more efficient substrate uptake, are there other ways to predictably achieve the same outcomes independent of calcium? Selective growth of PHA storing biomass in activated sludge provided significant benefits. Engineering these outcomes in practice can help to make renewable resource activities widely available as part of waste management services.

In Chapter 5, it was also observed that even though the addition of calcium increase the production of PHA in different municipal WWTPs, but not all them reached PHA contents of up to 0.6 gPHA/gVSS. This is likely due to the differences observed in the degree of enrichment for PHA-storing biomass, as discussed in Chapter 4. A higher initial degree of enrichment will allow to reach higher PHA contents in a shorter period of time. If the PHA-storing biomass fraction will double its size every 24 h, as observed with Bath activated sludge, longer accumulation times would be required to reach these maximum PHA contents. The effect of the initial degree of enrichment for PHA-storing biomass on the final biomass PHA content as function of the biomass growth response is illustrated in Figure 7.1. Similarly, it remains to be seen if the addition of calcium is beneficial to PHA producing enrichment cultures, as much higher productivities could be obtained in these cases.



## 7.3. Challenges for scaling-up mixed culture PHA production

In Chapter 2, it was shown that the technology for mixed culture PHA production is ready to be scaled up from pilot scale. Efforts to scale up the technology are coming from different companies and partnerships along Europe. Companies like Paques Biomaterials (https://www.paquesbiomaterials.nl/) and Venvirontech (https://www.venvirotech.com/) in The Netherlands and Spain, respectively, or partnerships like the Circular Biocarbon project (https://circularbiocarbon.eu/) are increasingly daring to make next steps and expect to reach commercially activities with copolymer PHBV supply chains in the coming years. In this process, chal-

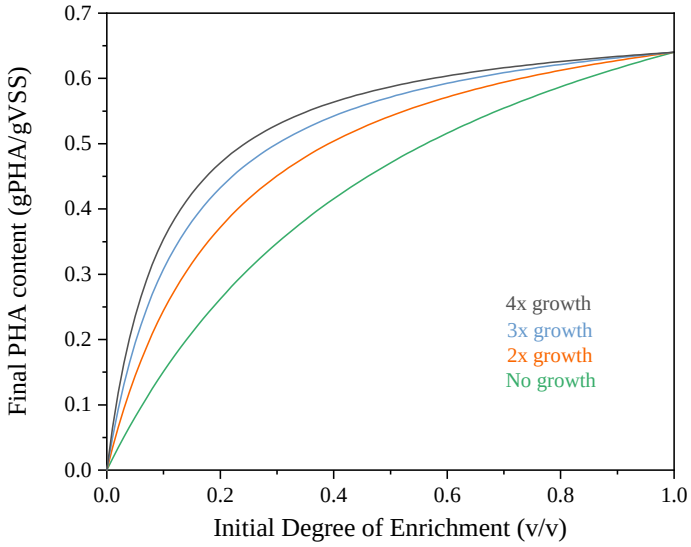


Figure 7.1. Final biomass PHA contents as function of the initial degree of enrichment for PHA-storing biomass in the initial municipal activated sludge at different growth rates (no growth or biomass increases two, three or four fold.



lenges are expected to be related to establishing long term agreements in supplies of the waste streams that will be used for PHA production, to implementing suitable downstream polymer recovery methods, and marketing developments bringing value from applications. All these challenges require success in technology, logistics alongside a network of connected stakeholders from different public and private sectors working together.

Many different waste streams can be applied for PHA production (Rodriguez Perez et al., 2018). However, an easily fermentable wastewater with a higher COD content and limited nutrients would be preferred at the initial stages of scaling up. Even if an optimal wastewater is selected for the initial stages of scaling up, there is a lack of piloting experiences in the downstream process. It remains unknown for instance what is the best solvent to be used at industrial scale. In Chapter 2, it was proposed to tune the downstream process depending of the application of the polymer. For this, it is necessary to connect different stakeholders from different sectors. Application developers and downstream engineers should sit together

and design a downstream process that can fulfill each other requirements. For this collaboration to be successful, enough material needs to be available so application developers can perform tests. This can be achieved with demonstration plants such as the one recently opened in the Netherlands as part of the PHA2USE project. This plant is expected to deliver enough materials to application developers and motivate for further scaling up. If these tests are successful, the first full scale installation for mixed culture PHA production is expected before 2030.

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# Epilogue

*”La rayuela se juega con una piedrita que hay que empujar con la punta del zapato. Ingredientes: una acera, una piedrita, un zapato, y un bello dibujo con tiza, preferentemente de colores. En lo alto está el Cielo, abajo está la Tierra, es muy difícil llegar con la piedrita al Cielo, casi siempre se calcula mal y la piedra sale del dibujo. Poco a poco, sin embargo, se va adquiriendo la habilidad necesaria para salvar las diferentes casillas (rayuela caracol, rayuela rectangular, rayuela de fantasía, poco usada) y un día se aprende a salir de la Tierra y remontar la piedrita hasta el Cielo, hasta entrar en el Cielo, lo malo es que justamente a esa altura, cuando casi nadie ha aprendido a remontar la piedrita hasta el Cielo, se acaba de golpe la infancia y se cae en las novelas, en la angustia al divino cohete, en la especulación de otro Cielo al que también hay que aprender a llegar. Y porque se ha salido de la infancia se olvida que para llegar al Cielo se necesitan, como ingredientes, una piedrita y la punta de un zapato.”*

*”Hopscotch is played with a pebble that you move with the tip of your toe. The things you need: a sidewalk, a pebble, a toe, and a pretty chalk drawing, preferably in colors. On top is Heaven, on the bottom is Earth, it’s very hard to get the pebble up to Heaven, you almost always miscalculate and the stone goes off the drawing. But little by little you start to get the knack of how to jump over the different squares (spiral hopscotch, rectangular hopscotch, fantasy hopscotch, not played very often) and then one day you learn how to leave Earth and make the pebble climb up into Heaven, the worst part of it is that precisely at that moment, when practically no one has learned how to make the pebble climb up into Heaven, childhood is over all of a sudden and you’re into novels, into the anguish of the senseless divine trajectory, into the speculation about another Heaven that you have to learn to reach too. And since you have come out of childhood you forget that in order to get to Heaven you have to have a pebble and a toe.”*

Julio Cortázar, Rayuela (1963)

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# Acknowledgements

Life is growing up and learning how to say goodbye. It does not matter how pleasant the journey was, all good things must come to an end. It is now time to say goodbye, and most importantly, thank you. These last four years I have met many interesting people. From all these people I have learned at least something that, I hope, has made me a better scientist and/or at least, a better person.

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# Curriculum Vitæ

Ángel ESTÉVEZ ALONSO

Ángel Estévez Alonso was born on 7$^{th}$ January 1994 in Vigo, northwest of Spain. In 2012, he started his Bachelor degree in Chemical Engineering at the Universidade de Santiago de Compostela. During this time, he received a grant from the Spanish ministry that allowed him to join the Environmental Biotechnology group and study the enzymatic oligomerization of natural flavonoids.

After graduation, Ángel went on to study the Msc in Chemical and Bioprocess Engineering at the same university. During his Master and Bachelor program, he performed an internship at Gwangju Institute of Science and Technology (South Korea) and two Erasmus exchanges, one in Yıldız Technical University (Turkey) and another one at Delft University of Technology (The Netherlands). At TU Delft, he became fascinated with mixed culture biotechnology and got the opportunity to join as PhD candidate in a collaboration between TU Delft and Wetsus under the supervision of Alan Werker, Robbert Kleerebezem and Mark van Loosdrecht. His PhD project was focused on the use of municipal waste activated sludge for biopolymers production and the result of this work is shown in these pages.

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# List of Publications

6. **Estévez-Alonso, Á.**, Arias-Buendía, M., Pei, R., van Veelen, H.P.J., van Loosdrecht, M.C.M., Kleerebezem, R. and Werker, A., 2022, *Calcium enhances polyhydroxyalkanoate production and promotes selective growth of the polyhydroxyalkanoate-storing biomass in municipal activated sludge*. Water Research, **226**, 119259.

5. **Estévez Alonso, Á.**, Altamira-Algarra, B., Arnau-Segarra, C., van Loosdrecht, M.C.M., Kleerebezem, R. and Werker, A., 2022, *Process conditions affect properties and outcomes of polyhydroxyalkanoate accumulation in municipal activated sludge*. Bioresource Technology, **364**, 128035.

4. Pei, R.[1], **Estévez-Alonso, Á.**[1], van Loosdrecht, M.C.M., Kleerebezem, R., Werker, A., 2022 *Exploring the limits of polyhydroxyalkanoate production with municipal activated sludge*. Environmental Science & Technology, **56** (16), 11729-11738.

3. **Estévez-Alonso, Á.**, van Loosdrecht, M.C.M., Kleerebezem, R. and Werker, A., 2021. *Simultaneous nitrification and denitrification in microbial community based polyhydroxyalkanoate production*. Bioresource Technology, **337**, 125420.

2. **Estévez-Alonso, Á.**[1], Pei, R.[1], van Loosdrecht, M.C.M., Kleerebezem, R. and Werker, A., 2021. *Scaling-up microbial community based polyhydroxyalkanoate production: status and challenges*. Bioresource Technology, **327**, 124790.

1. Mulders, M., **Estévez-Alonso, Á.**, Stouten, G.R., Tamis, J., Pronk, M. and Kleerebezem, R., 2020. *Volatile fatty acid product spectrum as a function of the solids retention time in an anaerobic granular sludge process*. Journal of Environmental Engineering (US), **146(8)**, 04020091.

Microbes are the gears that sustain all forms of life in planet Earth and are expected to become key players in the transition towards a more sustainable society. In this thesis, microbes from sewage sludge are used for the production of polyhydroxyalkanoates, biodegradable polymers that have the potential to replace many of the petroleum-based plastics that are used nowadays.