Converting Wastewater Treatment Plants into Polyhydroxyalkanoate Production Factories

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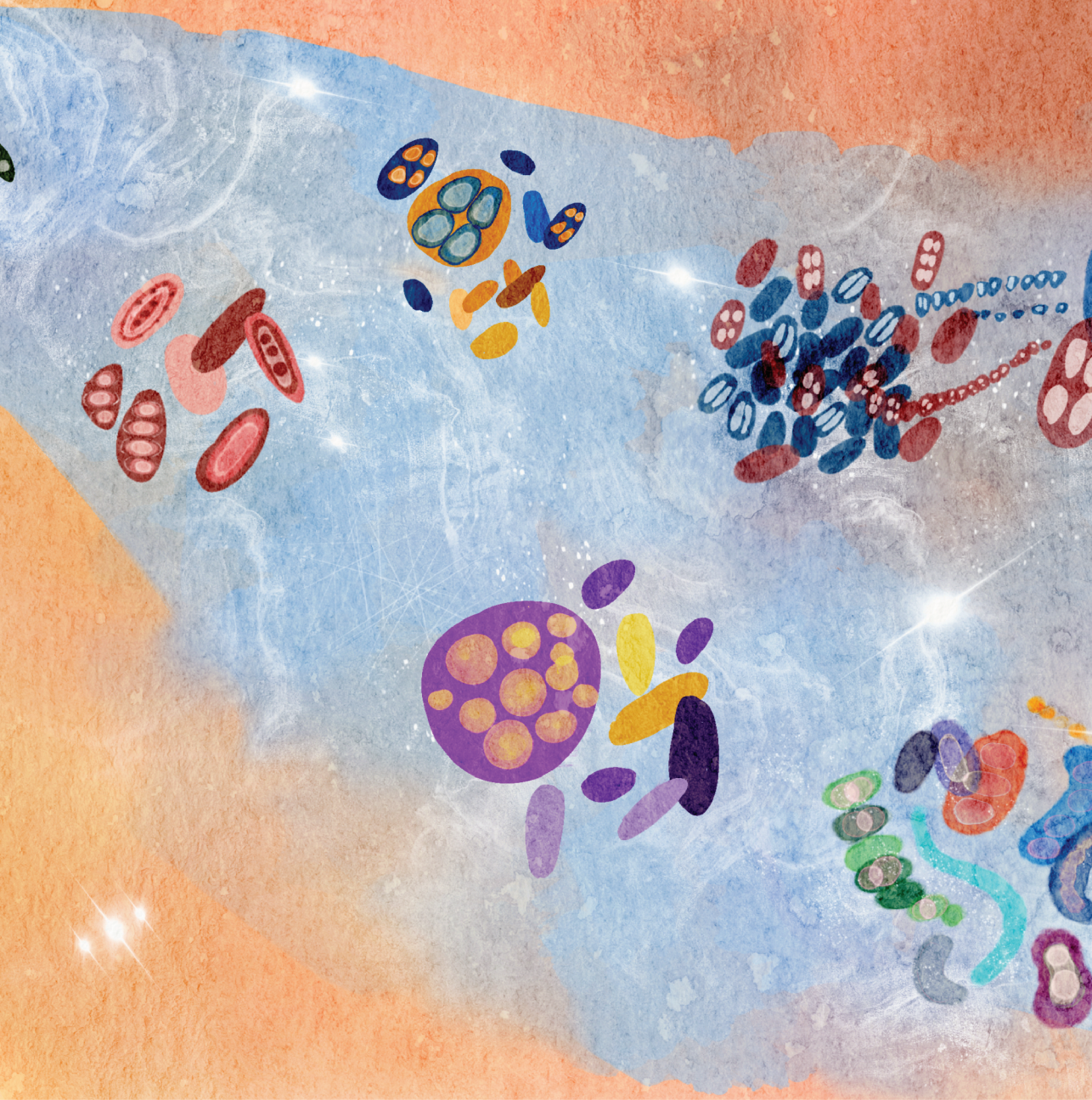
Ruizhe Pei



Propositions

accompanying the dissertation

Converting Wastewater Treatment Plants into Polyhydroxyalkanoate Production Factories

by

Ruizhe Pei

1. Current municipal wastewater treatment plants are selecting for common PHA accumulators but not for super accumulators. (*This thesis*)
2. The degree of enrichment by the end of a PHA accumulation process, starting with waste activated sludge, determines final biomass PHA content. (*This thesis*)
3. Acidic environments are the only way to preserve both the quantity and the quality of the PHA accumulated in municipal waste activated sludge. (*This thesis*)
4. Resource recovery from municipal wastewater treatment plant should not jeopardize the core service of protecting human health and maintaining a healthy ecosystem during wastewater treatment.
5. Online measurements and automation should be implemented to free PhD students from tedious labour work and enable more focus on conceptualizing and thinking.
6. The data mining top-down approaches like multi "Omics" in microbial ecology research and the associated funding distribution preferences make the bottom-up approach (isolation, characterization, building synthetic cultures) overlooked.
7. In scientific publication, reviewer comments should be transparent to the public and thus should be published alongside the manuscript.
8. Fossil-based products fuels the modern society.
9. Banning fossil-based products is the only way to build a sustainable society.
10. The individual eagerness to be superior goes against our parallel desire for equality in society.

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

Converting Wastewater Treatment Plants into Polyhydroxyalkanoate Production Factories

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 1 December 2022 at 12:30 o'clock

by

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This dissertation has been approved by the promotors.

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献给我的父母：高琪和裴延河。

Dedicated to my parents: Qi Gao and Yanhe Pei.

Contents

Summary

Fossils derived plastics offer a wide range of services in applications, from packaging to building and construction, to electronics and water distribution networks. The use of fossils to produce plastics releases the stored CO_2 and contributes to climate change. Due to durability and resistance to degradation, discarded plastics accumulate in the environment and affect the ecosystems. Polyhydroxyalkanoates (PHA) are considered as an alternative for bio-based and biodegradable plastics. PHA is a family of polyesters that is naturally synthesised by microorganisms. After extraction and purification, PHA shows thermoplastic properties similar to polypropylene and polyethylene. However, the current market share of all bioplastics in the overall plastic industry is small (3%) (Plastics Europe, 2020). Efforts are urgently needed to expand the global potential for greater capacity in bioplastics production. PHA may be accumulated directly using the waste activated sludge from wastewater treatment plants. Currently, methods and experiences for PHA accumulation directly using waste activated sludge are still at the pilot scale. This thesis critically evaluated the current status of the technology, identified knowledge gaps and then focused on deepening insight and fundamental understanding to help facilitate a scaling up to industrial scale.

Chapter 1 contains a general introduction about the context for the demand of waste derived PHA in current society. It explains the relevance and motivation for this thesis and then outlines the following research chapters.

Microbial community-based PHA production processes have been successfully applied up to the pilot scale. **Chapter 2** critically reviewed 19 pilot installations reported in the literature. Research at the pilot scale on microbial community-based PHA production is first categorised. Then the technology readiness was assessed with a focus on feedstocks, enrichment strategies, yields of PHA on the substrate, biomass PHA content and polymer characterisation. Subsequently, the challenges for further scaling-up of microbial community-based PHA production are identified. From the identified challenges, this thesis addressed the following research questions:

1. Why do different municipal activated sludge sources exhibit such different PHA accumulation potentials?

2. How does post accumulation process environment in downstream processing influence the fate of the PHA before its recovery from biomass?

The first research question was addressed by a progression of developments reported from **Chapters 3** to **5**. In **Chapter 3**, PHA was produced with waste activated sludge from municipal biological wastewater treatment. The distribution of PHA storage capacity was evaluated during the accumulation process at a pilot scale by methods of selective fluorescent staining with confocal laser scanning microscopy. Staining methods to visualise PHA granule distribution together with other specific biomass components (DNA, RNA and protein) were also developed and optimised. The optimised selective staining was applied to a specific activated sludge used for PHA accumulation as a case study. In the performed replicate PHA accumulation experiments at the pilot scale, biomass PHA contents of up to 0.48 gPHA/gVSS (n=3) were obtained. The PHA accumulation was visualised using the staining methods. Not all the biomass was engaged with the PHA storage activity for this activated sludge. Accumulated PHA granules were heterogeneously distributed within and between flocs in the biomass. These observations suggested that the PHA content for the bacteria storing PHA was significantly higher than the average PHA content measured for the biomass as a whole.

In **Chapter 4**, the selective staining methods were extended and validated quantitatively using the same PHA-rich biomass collected in **Chapter 3**. The image analysis quantified that the PHA-accumulating fraction of the activated sludge (degree of enrichment) in this case study was 55%. Thus even if the biomass PHA content reached 0.49 gPHA/gVSS (n=4), the accumulating fraction of the biomass was estimated to have the capacity to accumulate on average 0.60 gPHA/gVSS. The combination of quantitative microscopy and polymer mass assessment revealed that the degree of enrichment for PHA-storing bacteria and the average PHA storage capacity of the accumulating bacteria could affect the PHA accumulation potential for the given activated sludge.

To further elucidate what the determining factors for the biomass PHA content for different biomass are, an experiment similar with **Chapter 3** and **4** was performed using different sources of activated sludge. In **Chapter 5**, the established quantitative selective staining protocols were then applied together with a standardised *direct accumulation* assay on activated sludge collected from six different WWTPs. For each, it was estimated the maximum biomass PHA content, the degree of enrichment, and the average PHA content

in the PHA-storing biomass fraction. The degree of enrichment for PHA-accumulating bacteria was identified as the main reason different municipal activated sludge sources exhibit such different PHA accumulation potentials. It was found that the average PHA content within the PHA-accumulating biomass fraction was relatively constant at the level of 0.58 ± 0.07 gPHA/gVSS. This value is also considered a realistic target to be achieved when using municipal activated sludge for PHA production. The strategies to achieve this target should focus on obtaining a higher degree of enrichment either through selection during wastewater treatment or by selective growth during PHA accumulation.

Chapter 6 addresses the second research question, in sets of incubation experiments for the fate of exogenous and endogenous polyhydroxybutyrate (PHB). The PHB-rich activated sludge was evaluated over two weeks as a function of initial pH (5.5, 7.0 and 10) and incubation temperature (25, 37 and 55 °C), with or without aeration. PHA became consumed under aerobic conditions. Surprisingly, under anaerobic conditions, the accumulated PHB was consumed up to 63% (initial pH 7, 55 °C) within the first day. During incubation period that followed, biomass PHA content remained stable in the biomass. Lower degradation rates were found for acidic anaerobic incubation conditions at a lower temperature (25 °C). The PHB thermal properties were evaluated in the dried PHB-rich biomass and for the dimethyl carbonate extracted PHB film. The PHB-in-biomass melt enthalpy was indicative of the polymer extractability and was different for the different post-accumulation processes. An acidic post-accumulation environment led to higher melt enthalpies in the biomass and, consequently, higher extraction efficiencies. Considering both the quality and quantity of the polymer, acidic environments were found to be favourable for preserving the PHB accumulated in activated sludge.

Chapter 7 summarises the experience during the thesis preparation. It gives closing perspectives and implications on the research results. It also provides an outlook with recommendations on the ongoing developments and efforts for up-scaling PHA production directly coupled to wastewater treatment processes and services.

Samenvatting

Van fossiele brandstoffen afgeleide kunststoffen bieden een brede waaier aan toepassingen, gaande van verpakkingen over bouw en constructie tot elektronica en waterdistributienetwerken. Het gebruik van fossiele brandstoffen voor de productie van kunststoffen zorgt ervoor dat de opgeslagen CO_2 vrij komt en werkt zo klimaatverandering in de hand. Door hun duurzaamheid en weerstand tegen afbraak, hopen de afgedankte kunststoffen zich op in het milieu en tasten ze het ecosysteem aan. Polyhydroxyalkanoaat (PHA) werd beschouwd als een van de alternatieve biogebaseerde en biologisch afbreekbare kunststoffen. PHA is een familie van polyesters die op natuurlijke wijze door micro-organismen wordt gesynthetiseerd. Na extractie en zuivering vertoont PHA thermoplastische eigenschappen die vergelijkbaar zijn met die van polypropyleen en polyethyleen. Het huidige marktaandeel van alle bioplastics in de gehele plasticindustrie is echter klein (3%) (Plastics Europe, 2020). Er zijn dringend inspanningen nodig om de wereldwijde productiecapaciteit van bioplastics uit te breiden. PHA zou bijvoorbeeld rechtstreeks kunnen worden geaccumuleerd met behulp van actief slib uit afvalwaterzuiveringsinstallaties. Momenteel bevindt dit proces zich nog enkel op proefniveau. Dit proefschrift heeft de huidige status van deze technologie kritisch geëvalueerd, de hiaten in de kennis geïdentificeerd en zich vervolgens gericht op het verdiepen van inzicht en fundamenteel begrip om de opschaling naar de industriële schaal te vergemakkelijken.

Hoofdstuk 1 bevat een algemene inleiding over de context van de vraag naar PHA uit afval in de huidige maatschappij. Het verklaart de relevantie en motivatie voor dit proefschrift en schetst vervolgens de volgende onderzoekshoofdstukken.

Op microbiële gemeenschappen gebaseerde PHA productieprocessen zijn met succes toegepast op pilootschaal. In **hoofdstuk 2** zijn 19 in de literatuur gerapporteerde pilootprojecten kritisch bekeken. Onderzoek op pilootschaal naar op microbiële gemeenschappen gebaseerde PHA-productie wordt eerst gecategoriseerd. Vervolgens is de technologiegereedheid beoordeeld met de nadruk op grondstoffen, verrijkingsstrategieën, opbrengst van PHA op het substraat, PHA-gehalte van de biomassa en polymeerkarakterisering.

Vervolgens zijn de uitdagingen voor de verdere opschaling van de op microbiële gemeenschappen gebaseerde PHA-productie geïdentificeerd. Op basis van de geïdentificeerde uitdagingen werden in dit proefschrift de volgende onderzoeksvragen behandeld:

1. Waarom vertonen verschillende stedelijke actiefslibbronnen zulke verschillende PHA-accumulatiemogelijkheden?

2. Hoe beïnvloedt de post-accumulatieprocesomgeving van stroomafwaartse verwerking het lot van de PHA vóór het herstel uit biomassa?

De eerste onderzoeksvraag is beantwoord door een progressie van de ontwikkelingen die zijn gerapporteerd in de **hoofdstukken 3** tot en met **5**. In **hoofdstuk 3** is aangetoond dat PHA kan worden geproduceerd met afvalactief slib van biologische afvalwaterzuiveringsprocessen. De verdeling van de PHA-opslagcapaciteit werd geëvalueerd tijdens het accumulatieproces op een pilotschaal door methoden van selectieve fluorescente kleuring met confocale laserscanmicroscopie. Er werden ook kleuringmethodes ontwikkeld en geoptimaliseerd om de verdeling van PHA-korrels samen met andere specifieke biomassacomponenten (DNA, RNA en eiwitten) te visualiseren. De geoptimaliseerde selectieve kleuring werd toegepast op een specifiek actief slib dat gebruikt werd voor PHA-accumulatie als casestudy. In de uitgevoerde herhaalde PHA-accumulatie-experimenten op proefschaal werden PHA-gehaltes van de biomassa tot 0.48 gPHA/gVSS (n=3) verkregen. De PHA-accumulatie werd gevisualiseerd met behulp van de kleuringstechnieken. Niet alle biomassa was betrokken bij de PHA-opslagactiviteit voor dit actief slib. Geaccumuleerde PHA-korrels waren heterogeen verdeeld binnen en tussen vlokken in de biomassa. Deze waarnemingen suggereerden dat het PHA-gehalte voor de bacteriën die PHA opsloegen aanzienlijk hoger was dan het gemiddelde PHA-gehalte gemeten voor de biomassa als geheel.

In **hoofdstuk 4** zijn de selectieve kleuringmethoden uitgebreid en kwantitatief gevalideerd met gebruikmaking van dezelfde PHA-rijke biomassa die in **hoofdstuk 3** was verzameld. De beeldanalyse kwantificeerde dat de PHA-accumulatiefractie van het actief slib (verrijkingsgraad) in deze casestudy 55% bedroeg. Dus zelfs als het PHA-gehalte van de biomassa 0.49 gPHA/gVSS bereikte (n=4), werd geschat dat de accumulerende fractie van de biomassa het vermogen had om gemiddeld 0.60 gPHA/gVSS te accumuleren. Uit de combinatie van kwantitatieve microscopie en bepaling van de polymeermassa bleek dat de mate van aanrijking voor PHA-houdende bacteriën en de gemiddelde PHA-opslagcapaciteit van de accumulerende bacteriën van invloed kunnen zijn op het PHA-accumulatiepotentieel voor het gegeven actief slib.

Om verder te verduidelijken wat de bepalende factoren voor het PHA-gehalte van verschillende biomassa's zijn, werd een experiment uitgevoerd dat vergelijkbaar is met de **hoofdstukken 3** en **4**, waarbij verschillende bronnen van actief slib werden gebruikt. In **hoofdstuk 5** werden vervolgens de vastgestelde kwantitatieve selectieve kleuring protocollen toegepast samen met een gestandaardiseerde directe accumulatie assay op actief slib verzameld van zes verschillende RWZI's. Voor elk werd een schatting gemaakt van het maximale PHA-gehalte van de biomassa, de aanrijkingsgraad, en het gemiddelde PHA-gehalte in de PHA-opslagfractie van de biomassa. De verrijkingsgraad voor PHA-accumulerende bacteriën werd geïdentificeerd als de belangrijkste reden waarom verschillende bronnen van stedelijk actief slib zo'n verschillend PHA-accumulatiepotentieel vertonen. Er werd geconstateerd dat het gemiddelde PHA-gehalte binnen de PHA-accumulerende biomassafractie relatief constant was op het niveau van 0,58 ± 0,07 gPHA/gVSS. Deze waarde wordt ook beschouwd als een realistische doelstelling die moet worden gehaald bij het gebruik van gemeentelijk actief slib voor de productie van PHA. De strategieën om dit doel te bereiken moeten gericht zijn op het verkrijgen van een hogere verrijkingsgraad, hetzij door selectie tijdens de afvalwaterbehandeling, hetzij door selectieve groei tijdens de PHA-accumulatie.

Hoofdstuk 6 behandelt de tweede onderzoeksvraag. Hierbij werden reeksen van incubatieexperimenten naar het lot van exogeen en endogeen polyhydroxybutyraat (PHB) gebruikt. Het PHB-rijke actief slib werd gedurende twee weken geëvalueerd als functie van de initiële pH (5,5, 7,0 en 10) en de incubatietemperatuur (25, 37 en 55 °C), met of zonder beluchting. Onder aërobe omstandigheden werd PHA verbruikt. Verrassend was dat onder anaërobe omstandigheden de geaccumuleerde PHB binnen de eerste dag tot 63% werd verbruikt (pH 7, 55 °C). Gedurende de daaropvolgende incubatieperiode bleef het PHA-gehalte in de biomassa stabiel. Lagere afbraaksnelheden werden gevonden voor zure, anaerobe incubatieomstandigheden bij een lagere temperatuur (25 °C). De thermische eigenschappen van PHB werden geëvalueerd in de gedroogde PHB-rijke biomassa en voor de met dimethylcarbonaat geëxtraheerde PHB-film. De PHB-in-biomassa smeltenthalpie was indicatief voor de extraheerbaarheid van het polymeer en was verschillend voor de verschillende post-accumulatieprocessen. Een zuur post-accumulatie milieu leidde tot hogere smelt enthalpie in de biomassa en, bijgevolg, een hogere extractie efficiëntie. Voor zowel de kwaliteit als de kwantiteit van het polymeer bleken zure milieus het meest gunstig te zijn voor het behoud van het in actief slib geaccumuleerde PHB.

Hoofdstuk 7 geeft een samenvatting van de ervaringen tijdens de voorbereiding van het proefschrift. Het geeft afsluitende perspectieven en implicaties op de onderzoeksresulta-

ten. Het geeft ook een vooruitblik en aanbevelingen over de lopende ontwikkelingen en inspanningen om de productie van PHA direct gekoppeld aan afvalwaterzuiveringsprocessen en -diensten op te schalen.

摘要

化石衍生塑料被广泛应用于包装，建筑和施工，电子和配水网络等领域。使用化石原料生产塑料制品会释放二氧化碳并导致气候变化。并且由于塑料的耐用性和抗降解性，废弃的塑料会在环境中积累并对生态系统造成负面影响。聚羟基链烷酸酯(PHA)被认为是可替代传统塑料的生物基可生物降解塑料之一。PHA是由微生物天然合成的一类聚酯。经过提取和纯化后，PHA显示出类似于聚丙烯和聚乙烯的热塑性。然而，目前生物基塑料在整个塑料行业中的市场份额很小（3%）(Plastics Europe, 2020)。目前迫切需要努力提高全球范围内生物塑料生产的能力。PHA可以直接使用来自污水处理厂的废弃活性污泥进行积累。然而目前，直接利用废活性污泥进行积累PHA仍处于中试规模。本论文批判性地评估了该技术的现状，确定了知识缺口，然后专注于加深基本理解，以促进向工业规模的扩展。

第**1** 章概述了当前社会对废物衍生PHA的需求背景。它解释了本文的相关性和动机，然后概述了以下研究章节。

基于微生物群落的PHA生产生产工艺已成功应用于中试规模。第**2** 章批判性地回顾了文献中报道的19个中试装置。首先对基于微生物群落的PHA生产的中试规模研究进行了分类。然后评估了技术准备情况，重点是原料、富集策略、基于基质上的PHA产量、PHA含量和聚合物表征。随后，确定了进一步扩大基于微生物群落的PHA生产规模的挑战。从确定的挑战中，本论文探讨了以下研究问题:

1. 为什么不同的市政活性污泥源表现出不同的PHA积累潜力?

2. 积累工艺之后的中下游加工的环境条件如何影响提取之前的PHA?

第**3** 章至第**5** 章回答了第一个研究问题。在第**3** 章中，PHA可以用生物废水处理过程中产生的废弃活性污泥生产。在中试规模的积累过程中，通过使用选择性荧光染色共聚焦激光扫描显微镜进行的方法，对PHA存储容量的分布情况进行了评估。在此基础上还开发并优化了染色方法将PHA颗粒的分布与其他特定的生物质成分（DNA、RNA和蛋白质）一起可视化的染色方法。作为案例研究，优化后的选择性染色被应用于用于一种用来进行PHA积累的特定活性污泥中PHA。中试规模重复

的PHA积累实验获得了高达0.48 gPHA/gVSS（n=3 的PHA含量。PHA的积累是用染色方法来观察的。结果显示并非所有的生物质都与这种活性污泥的PHA储存活动有关。积聚的PHA颗粒在生物质中的絮状物内和絮状物之间分布不均匀。这些观察结果表明，PHA积累细菌的PHA含量显著高于对整个生物质测量的平均PHA含量。

在第**4** 章中，使用第**3** 章中收集的同样的富含PHA的生物质，对选择性染色方法进行了扩展和定量验证。图像分析量化了本案例研究中活性污泥的PHA积累部分（富集度）为55%。因此，尽管生物质总体PHA含量为0.49 gPHA/g（n=4），但估计生物质的积累部分平均具有积累0.60 gPHA/gVSS 的能力。定量显微镜和聚合物质量评估的结合显示，PHA储存细菌的富集程度和储存细菌的平均PHA储存能力可以影响定活性污泥的PHA积累潜力。

为了进一步阐明不同生物质的PHA 含量不同的决定因素是什么，不同来源的活性污泥被用来进行了与第**3** 章和第**4** 章类似的实验。在第**5** 章中，将已建立的定量选择性染色方案与标准化的直接累积试验一起应用在了从六个不同污水处理厂收集的活性污泥中。对每种污泥都估计了PHA的最大含量、富集程度以及储存PHA的生物质部分的平均PHA含量。PHA积累细菌的富集程度被认为是不同的城市活性污泥来源表现出不同的PHA积累潜力的主要原因。研究发现发现PHA 积累生物质部分内的平均PHA含量相对恒定在0.58±0.07 gPHA/gVSS的水平。这一数值也被认为是使用城市活性污泥生产PHA时能达到的现实目标。实现这一目标应侧重于通过在废水处理过程中的选择或在PHA积累过程中的选择性生长来获得更高的富集程度。

第**6** 章讨论了第二个研究问题。在有或没有曝气的情况下，评估了富含PHB的活性污泥作在不同初始pH 值（5.5、7.0 和10）和培养温度（25、37 和55 °C）。PHB在有氧条件下被消耗。令人惊讶的是，在厌氧条件下，积累的PHB在第一天就被消耗了高达63%（初始pH值为7，55 °C）。在接下来的培养期间，生物质中的PHA含量保持稳定。在较低温度的酸性厌氧培养条件下，PHB降解率较低（25 °C）。我们也对富含PHB的干燥生物质和碳酸二甲酯提取的PHB薄膜中的PHB热性能进行了评估。PHB在生物质中的熔融焓值表明了聚合物的可提取性，并且在不同的蓄积后过程中是不同的。酸性环境下生物质中的PHB熔融焓较高，因此提取效率也较高。考虑到聚合物的质量和数量，酸性环境有利于保护活性污泥中积累的PHB。

第**7** 章总结了论文准备过程中的经验。它对研究结果给出了封闭的观点和启示。它还就与废水处理工艺和服务直接相关的PHA生产规模的持续发展和努力提供了展望和建议。

1

Introduction

1.1. Petroleum and Modern Society

The fossils of ancient life, plants, higher organisms and microorganisms, formed petroleum after becoming stored underground and subjected to high temperatures and pressures over millions of years. Petroleum forms the backbone of our modern society. It is a primary energy source that maintains us in our daily life, from households, to factories, and transport. Petrochemicals are organic compounds derived from petroleum that also shape modern society. They are the chemical building blocks essential for producing plastics, clothing, detergents, and so forth. From petrochemicals, different plastic materials, such as polypropylene (PP), polyethene (PE), and polyvinyl chloride (PVC), can be produced. In 2020, 367 Mt tons of plastics were produced worldwide (Plastics Europe, 2021). These plastics offer a wide range of services in applications, from packaging, to building and construction, to electronics and water distribution networks. These applications rely on the critical material property specifications of plastics that include as ease of processing into parts and articles, light weight with strength and durability, and resistance to degradation.

From all plastics produced in 2015, it was estimated that only 17.4% was recycled, 23.4% was incinerated, and 59.2% was discarded (Geyer et al., 2017). For discarded plastics without proper treatments (i.e. landfills) the advantages of plastics (i.e. durability and a slow degradation rate) cause issues. They can stay in our ecosystems for thousands of years. The accumulation of plastics in the environment can lead to problems of soil toxicity, leaching of chemicals, fish suffocation, and damage to all flora, fauna, and humans. Petroleum is fossil organic carbon-rich matter. Its extraction and use leads furthermore to an excess of carbon dioxide released faster than its rate of assimilation. An excess build-up of carbon dioxide in the atmosphere contributes to global warming and climate change. Therefore, there is need for a transition to greater use of renewable rather than fossil organic rich matter. Bio-based plastics can be produced using non-fossil organic residuals and waste as feedstock. Different bio-based polymers as platforms for plastics such as polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) can be produced. These bioplastics can also be made to be completely biodegradable. Presently, 97% of plastics are produced with petroleum, and only 3% of all plastics produced are bioplastics (Plastics Europe, 2020). Therefore, efforts are urgently needed to enable expanded global potential for greater generic capacity in bioplastics production.

1.2. Wastewater Treatment Plants

A milestone of development for society in human history has been the implementation of wastewater treatment plants (WWTPs). These facilities have improved quality of life with community health and environment protection (Lofrano et al., 2010). Sewage systems prevented cross contamination between drinking water intake and wastewater discharge, to ensure sustained access to an essential resource, potable water. Pollutant removal during wastewater treatment prevents environmental issues, for example, eutrophication. Modern day WWTPs often apply primary treatment to remove readily separable particulate matter, followed by a secondary biological treatment. One of the most used secondary treatment technologies is the activated sludge process. In this biological process, a bacterial biomass is grown in order to remove contaminants including dissolved organic carbon and inorganic nutrients. After secondary treatment, tertiary treatment may be applied as a polishing step like advanced chemical treatment or disinfection before effluent discharge. Wastewater treatment can be energy intensive and produces by-products that need further management. Primary treatment produces an organic sludge (primary sludge). Secondary treatment produces a bacterial biomass rich waste activated sludge (secondary sludge). Traditionally, waste sludge has been managed as a waste by-product for disposal. Typically in The Netherlands, the combined organic rich primary and secondary thickened and dewatered sludge is reduced for its final disposal by anaerobic digestion and incineration.

With this in mind, advancements for wastewater process configurations aim for more effective treatment methods, smaller footprints, energy neutral operations, and resource recovery. Akin to organic rich petroleum, organic rich waste sludge can be a resource for energy and platform chemicals. In the past decades, more research has focused on potentials of resource recovery from the WWTPs in addition to demands of wastewater treatment before effluent discharge (Kehrein et al., 2020). From WWTPs, energy can be recovered from anaerobic digestion for biogas and incineration of residual organic matter. The WWTPs could also serve as production facilities for renewed and recycled chemicals and minerals. Organic carbon, nitrogen, and phosphorus are the main components to be recovered and valorised. Organic carbon can be recovered as volatile fatty acids (VFAs) through acidogenic fermentation of primary or secondary sludge. The VFAs are a platform chemical that can be separated or further transformed into a methane rich biogas. Nitrogen and phosphorus may also be recovered from sludge and then reused. Resources recovery not only provides valued raw materials but also raises the value for operating WWTPs for society. This value could improve the overall benefits of WWTP

ecosystem services with regional sustainability. In 2020, United Nations estimated that only 55.5% of all wastewater was treated. Improving the value for WWTPs could help to motivate wider gains as further incentive to achieve the United Nations Sustainable Development Goal: Clean Water and Sanitation.

1.3. Polyhydroxyalkanoate Production using Wastes

PHAs are a family of polyesters that accumulates intracellularly due to a growth imbalance for many species of bacteria. PHA granules are stored in dynamically changing environments when, for example, surplus organic carbon substrates are present but growth is constrained by factors such as limited availability of nitrogen and/or phosphorus (Majone et al., 1999; Reis et al., 2003; Van Loosdrecht et al., 1997). PHA usually accumulates as a high molecular weight polymer (circa 2300 kDa), and it accumulates in the cytoplasm as insoluble inclusion bodies enclosed by membranes and embedded proteins, and with a diameter in the range of 200 to 500 nm (Bengsston et al., 2017; Majone et al., 2014; Morgan-Sagastume et al., 2014; Ortelli et al., 2019; Patel et al., 2009). A PHA molecule usually consists of 600 to 35,000 monomers, and the monomer side chain is typically a saturated alkyl group (Tan et al., 2014).

PHAs serve as energy and carbon reserves for growth when all other growth factors are available, and they are naturally synthesised and stored by different types of microorganisms (Dawes et al., 1973). As such PHAs are inherently biodegradable in all microbiologically active environments (soils, marine and freshwater, etc.) at rates depending on the polymer form (powder, film, crystallinity, surface quality, etc.), additional chemistry, and the exposure environment (El-Hadi et al., 2002; Jendrossek et al., 2002; Zaheer et al., 2018). As polymers, PHAs shared thermoplastic properties similar to polypropylene and polyethylene. The monomer composition as defined by the alkyl groups for a type of PHA results in a wide range of possible physico-chemical and mechanical properties. Consequently, depending on the PHA-type and associated property specifications, a wide variety of processing methods are possible for riches of opportunities in commercial applications, e.g. packaging, disposable items and/or biodegradable carriers (Raza et al., 2018). However, in the relatively small global bioplastics market PHA is still only a minor player with 1.4% (2018) of volume share. PHA can be an alternative source of polymer for plastics applications. Due to the current worldwide plastics demand, ambitions need to be realistic with goals to replace petroleum-based plastics. Supply chains need to match market needs and in ways that may not directly compete, at first. To start, PHAs can offer

services where bio-based and biodegradable properties make sense (Bauchmüller et al., 2021).

Currently, PHAs are produced commercially with pure culture fermentation processes using high purity substrates and sterile starting conditions to avoid contaminating microbes. Defined strains can accumulate PHAs intracellularly up to 90% of their cell dry weight (Koller, 2018). The strict requirements for the substrates and culturing conditions for commercial production results in high production costs (Raza et al., 2018). In the pure culture approach, an isolated world is created for a specific microorganism. Alternatively, in the past decades, efforts have been made to exploit the microbial-community based approaches. These approaches engineer the process environments to apply an inherent selection pressure for culturing phenotype of PHA-accumulating microorganisms and avoiding unwanted microorganisms (Kleerebezem et al., 2007). Thus, non-sterilized VFAs-rich substrates can be generated from waste stream fermentation and used as a feedstock for PHA production. Avoiding sterilization costs and using waste organic residuals as feedstocks are ways to lower overall PHA production costs.

Microorganisms that can accumulate PHA thrive under dynamic environmental conditions. Consequently, it has been shown that engineered alternating presence and absence of a carbon source, so-called feast-famine bioprocesses, can be used to selectively grow PHA-accumulating microorganisms (Kourmentza et al., 2017).

Feast-famine selection can be applied purposefully in a biomass production reactor or it could be inherent in existing secondary wastewater treatment facilities. The purposefully applied feast-famine regime is used in the *enrichment accumulation* approach. This approach applies optimum selective pressures in an enrichment step to produce functional biomass with a high degree of enrichment for PHA-accumulating microorganisms. Subsequently, a VFA-rich stream is fed to the functional biomass to maximise the biomass PHA content in the PHA accumulation process. At laboratory scale *enrichment accumulation* can achieve a biomass PHA content up to 90% cell dry weight (Johnson et al., 2009).

The feast-famine conditions are found in activated sludge bioprocesses of current WWTPs due to configuration or diurnal loading patterns that result in a dynamic cycle of substrate supply (Van Loosdrecht et al., 1997). Due to these dynamic conditions, waste activated sludge from municipal wastewater treatment is also enriched with the PHA-storing phenotype. Waste activated sludge can be used directly as the functional biomass without further enrichment for PHA accumulation in the *direct accumulation* approach. Compared to *enrichment accumulation*, *direct accumulation* approach can also reach

significant biomass PHA contents (Anterrieu et al., 2014; Bengsston et al., 2017; Bengtsson et al., 2017; Á. Estévez-Alonso et al., 2021a; Morgan-Sagastume et al., 2015; Morgan-Sagastume et al., 2014; Reis et al., 2003). The *direct accumulation* approach allows for exploitation of an already produced waste activated sludge for resource recovery from WWTPs.

After the PHA accumulation process, PHA needs to be extracted and purified through downstream processing (DSP). A typical DSP process includes cell harvesting, pretreatment, PHA extraction and purification (Kourmentza et al., 2017). Harvesting aims to separate the PHA-rich biomass from the culture through filtration or centrifugation. Then, pretreatment aims to stabilise the PHA in the biomass by thermal drying or lyophilization. After that, the extraction step aims to recover the PHA using methods ranging from chemical, physical, mechanical to biological techniques. Afterwards, depending on the specific purity requirements, the polymer can be washed or polished. DSP steps are essential for the preservation of polymer quantity and quality. DSP is also critical to the overall process economics (Kourmentza et al., 2017; Werker et al., 2020).

For microbial-community based PHA production process, VFAs-derived organic substrates are often used. The VFAs spectrum determines the type of the PHAs that can be produced (Jiang et al., 2011). The most common polymers are a blend of hydroxybutyrate and hydroxyvalerate monomers with different ratios. The different hydroxyvalerate content leads to different polymer physico-chemical properties (i.e. crystallinity, brittleness etc.) and ultimately different applications.

Compared to the commercial pure culture PHA production (25.3 ktPHA/yr), microbial-community based PHA production by enrichment or *direct accumulation* approaches currently moving from pilot scale (~ 0.5 kgPHA/day) to demonstration scale (~ 37.5 kgPHA/day) (Vandi et al., 2018). Research effort is still needed to support scaling-up the microbial-community based PHA processes for industrial production. It is a goal that the organic rich sludges from wastewater can be a biomass source for producing bioplastics in the future. If one could generically expand the methods and wider potential for commercial PHA production, then we may get one step closer to a more sustainable society.

1.4. Outline of the Thesis

This thesis research focused on deepening insight and fundamental understanding of the *direct accumulation* approach for producing PHA. Waste activated sludge from municipal wastewater treatment processes has been shown to be a good biomass source for PHA production (Bengsston et al., 2017; Á. Estévez-Alonso et al., 2021a). However, activated sludge by its nature is a complex biomass with diversity of microbial species, as well as both biodegradable and non-biodegradable (inert) solids. Therefore, much effort in this work has been devoted to unravelling the complexity towards understanding what factors may influence how much PHA is produced in *direct accumulation*, and the preservation of the PHA produced just after the accumulation process is completed. An ambition was to understand how different activated sludge sources can reach the maximum possible PHA accumulation potential and to develop relevant industrial scale strategies for post-processing PHA-rich biomass.

The principal research questions in the work were as follows:

1. Why do different municipal activated sludge sources exhibit such different PHA accumulation potentials?

2. How does the post accumulation process environment in DSP influence the fate of the PHA before its recovery from biomass?

These research questions are addressed in the following five chapters. **Chapter 2** reviews the current status of microbial community-based PHA production at pilot scale, and the challenges for the technology up-scaling. The first research question was addressed by a progression of developments reported from **Chapters 3** to **5**. As a first step, in **Chapter 3**, selective staining methods were developed and implemented qualitatively to monitor a *direct accumulation* process. In **Chapter 4**, the selective staining methods were then extended and validated quantitatively in order to probe the degree of enrichment for PHA-accumulating microbes and average PHA accumulation capacity as determining factors. The established quantitative selective staining protocols were then applied in a standardised *direct accumulation* assay on activated sludge collected from six different WWTPs. The degree of enrichment for PHA-accumulating microbes and their PHA accumulation capacity were estimated for each. Developments in progression from **Chapters 3** to **5** allowed an estimation of the limits for *direct accumulation* and the identification of strategies to maximise the biomass PHA content using waste activated sludge. The second research question was addressed in **Chapter 6**. **Chapter 6** embraces sets of experimental evaluations for the fate of exogenous and endogenous PHA. PHA-rich activated

sludge requires downstream processing, and the stability of stored PHA has not been systematically evaluated as a function of different environmental conditions for industrial scale developments. **Chapter 7**, provides closing perspectives on the research results and their implications with outlook and recommendations in the ongoing developments and efforts to up-scaling PHA production directly coupled to wastewater treatment processes and services.

2

Scaling-up Microbial Community-based Polyhydroxyalkanoate Production: Status and Challenges

Lab Scale
1.5 L
<5 g PHA/d

Pilot Scale
160 L
<500 g PHA/d

Pre-test
4 m^3
~ 15 kg PHA/d

Demo
10 m^3
~ 37.5 kg PHA/d

[1]This chapter has been published: Estévez-Alonso, Á., Pei, R., van Loosdrecht, M. C., Kleerebezem, R., & Werker, A. (2021a). Scaling-up microbial community-based polyhydroxyalkanoate production: status and challenges. *Bioresource Technology*, *327*, 124790. https://doi.org/10.1016/j.biortech.2021.124790.

[2]Photos by Ángel Estévez-Alonso, Erik de Vries, Paques Biomaterials BV, PHA2USE in the order of appearance.

Highlights:

- *Microbial community-based PHA production outcomes at pilot-scale are reviewed.*
- *Challenges to scale-up relate to linking context between process and application.*
- *Niche applications that can meet process and commercial demands are presented.*
- *Research needs and practical steps forward are identified and discussed.*

Abstract
Conversion of organic waste and wastewater to polyhydroxyalkanoates (PHAs) offers a potential to recover valuable 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 the scientific 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, yields of PHA on substrate, biomass PHA content and polymer characterization. From this assessment, the challenges for further scaling-up of microbial community-based PHA production are identified.

Keywords: *polyhydroxyalkanoates (PHAs), biopolymer, pilot-scale, resource recovery, organic waste*

2.1. Introduction

Nowadays, as society motivates goals of developing circular economies, converting waste into valuable raw materials is increasingly drawing 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, 2020b). 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 produce a significant mass of waste activated sludge (WAS). The WAS is typically incinerated (with or without pre-treatment 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 et al., 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 was 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 et al., 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 (Majone et al., 1999; Reis et al., 2003; Van Loosdrecht et al., 1997). PHAs are not soluble in water, therefore they are accumulated by bacteria as cytoplasmic intracellular granules forming inclusion bodies (Majone et al., 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, so-called 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 with an estimated market value of 7.0 US$/kg (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 et al., 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 first to produce volatile fatty acids (VFAs) that are subsequently metabolized by the 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., 2009).

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 (Chanprateep, 2010; Dias et al., 2006; Kourmentza et al., 2017; Valentino et al., 2017; Verlinden et al., 2007). 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. Pilot-scale installations enable the production of large amounts of PHAs making it possible to start to address quality in the context of commercial applications Table 2.1. Additionally, a pedigree of life cycle assessment studies have repeatedly concluded that there is a clear beneficial impact of microbial community-based produced PHA over petroleum-based polymers (Bengtsson et al., 2017; Fernández-Dacosta et al., 2015; Gurieff et al., 2007; Harding et al., 2007; Heimersson et al., 2014; Morgan-Sagastume et al., 2016; Yadav et al., 2020). Despite these

successful pilot plant operations to date and positive indications for environmental performance, breakthrough with a demonstration of commercial full-scale PHA production is still not yet realized.

This work aims to review the current status of microbial community-based PHA production, with emphasis on the collective experience gained from work at pilot-scale and, from those experiences, identify bottlenecks that present challenges to come on the road to realize full-scale production of commercial quality 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 (Dietrich et al., 2017; Kourmentza et al., 2017; Li et al., 2020; Nikodinovic-Runic et al., 2013; Rodriguez Perez et al., 2018; Sabapathy et al., 2020; Valentino et al., 2017).

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; Moretto et al., 2020b; Mulders et al., 2020a; Tamis et al., 2014a; Tamis et al., 2018; Valentino et al., 2018; Valentino et al., 2019a; Valentino et al., 2019b). After the functional biomass is produced, the same pre-fermented VFA-rich stream is most often used with appropriate modulation of nutrient 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 WAS for PHA production. This approach is based on the subsequent exploitation of WAS produced as a by-product from treating wastewater in a WWTP. A WAS with significant PHA accumulation potential can

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	-

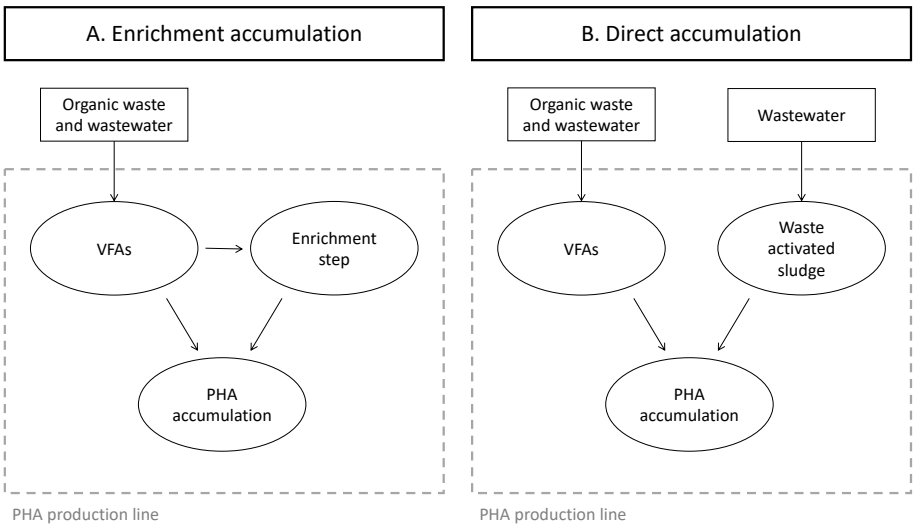

Figure 2.1: Critical defining differences between the *enrichment accumulation* and the *direct accumulation* approaches. Adapted from Bengtsson et al. (2017).

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; Bengsston et al., 2017; Bengtsson et al., 2017; Conca et al., 2020; Larriba et al., 2020; Morgan-Sagastume et al., 2015; Morgan-Sagastume et al., 2014). 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 WAS gives the opportunity to integrate PHA production processes into the municipal wastewater treatment as an extension to present day sludge management

goals (Bengsston et al., 2017). However, other supplies of (waste) organic feedstock are required in order to be able to exploit the polymer storing potential of the WAS 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 gVFA-COD/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., 2009; Palmeiro-Sánchez et al., 2016). 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 (Bengsston et al., 2017; Tamis et al., 2014a), fermented paper mill wastewater (Tamis et al., 2018) and starch-rich wastewater (Morgan-Sagastume 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 (Moretto et al., 2020a; Moretto et al., 2020b; Mulders et al., 2020a; Valentino et al., 2018; Valentino et al., 2020; Valentino

et al., 2019a; Valentino et al., 2019b), fermented tomato waste (Bengtsson et al., 2017) and fermented primary sludge (Bengsston et al., 2017; Conca et al., 2020; Morgan-Sagastume et al., 2015). To cope with suspended solids, a solid-liquid separation stage for the feedstock prior entering the PHA production line has been evaluated (Moretto et al., 2020a; Moretto et al., 2020b; Valentino et al., 2018; Valentino et al., 2020; Valentino et al., 2019b). Suspended solids for PHA accumulation feedstocks after fermentation were reduced to levels in the order of 0.02 gTSS/gsCOD by drum filtration (Bengsston et al., 2017). 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 (Bengsston et al., 2017). 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 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.

Table 2.2: Feedstocks used for the PHA accumulation reactors.

Feedstock	TS gTS/L	VS gVS/L	sCOD gCOD/L	VFA/sCOD -	C2 %	C3 %	C4 %	C5 %	NH_4^+ mgN/L	PO_4^- mgP/L	Alkalinity $mgCaCO_3/L$	pH -	References
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., 2017)
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	(Bengsston et al., 2017)
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	
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)

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

Continuation of **Table 2.2**.

Feedstock	TS gTS/L	VS gVS/L	sCOD gCOD/L	VFA/sCOD -	C2 %	C3 %	C4 %	C5 %	NH_4^+ mgN/L	PO_4^- mgP/L	Alkalinity $mgCaCO_3/L$	pH -	References
Acetic acid	n.a.	n.a.	-	100	100	0	0	0	0	0	-	-	(Valentino et al., 2019b)
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	
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	-	-	(Conca et al., 2020)
Fermented cellulosic primary sludge liquid	-	-	8.8 ± 1.6	94	24	50	12	9	326 ± 23	70 ± 12	-	4.8 ± 0.1	
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	-	-	(Valentino et al., 2020)
Fermented OFMSW and SAS mix	n.d.	n.d.	27 ± 4	85 ± 9	-	-	-	-	673 ± 72	119 ± 11	-	-	
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	-	-	(Morgan-Sagastume et al., 2020)
Acetic/propionic acid	n.a.	n.a.	9-100	100	C2+C4: 60-100		C3+C5: 0-40		1000	50	-	5.6	
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.

2.2.2. Yields of PHA on Substrate

Yields on substrates are defined as the amount of PHA produced per gram of consumed VFA (gPHA-COD/gVFA-COD) 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., 2009). 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 gPHB-COD/gButyrate-COD compared to 0.75 gPHB-COD/gAcetate-COD 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 gPHA-COD/gVFA-COD were observed when synthetic or nutrient-poor streams were used as feedstocks (Conca et al., 2020; Tamis et al., 2014a; Tamis et al., 2018; Valentino et al., 2018; Valentino et al., 2019b). However, when nutrients-rich streams were applied, a wider range with lower average yields have been reported, 0.33-0.61 gPHA-COD/gVFA-COD (Moretto et al., 2020a; Moretto et al., 2020b; Valentino et al., 2018; Valentino et al., 2020; Valentino et al., 2019b). 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 gPHA-COD/gVFA-COD while working with leachate from OFMSW, a nutrients-rich and acetate-rich waste stream (0.44 ± 0.11 gAcetate-COD/gVFA-COD). 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 gPHA-COD/gVFA-COD. Consistent yields are reported within the same work (<10% error), however large differences 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 gPHA-COD/gVFA-COD (Anterrieu et al., 2014; Bengsston et al., 2017; Bengtsson et al., 2017; Conca et al., 2020; Morgan-Sagastume et al., 2015; Morgan-Sagastume et al., 2020). An acclimation to the feedstock has been reported to improve the performance of direct accumulation (Morgan-Sagastume et al., 2017). 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 (Bengsston et al., 2017; 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 et al., 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 (Mulders et al., 2020b; Valentino et al., 2015). Sustained selective growth under conditions of partial phosphorus 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 accumulation* or the *direct accumulation* approaches were used. Overall it can be concluded that specific feedstock dependent pre-treatment 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; Tamis et al., 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 (Bengsston et al., 2017; 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 (Valentino et al., 2019b), 25-28°C (Moretto et al., 2020a; Moretto et al., 2020b) and 30 ± 2°C (Mulders et al., 2020a; Tamis et al., 2014a; Tamis et al., 2018).

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 Acetic acid/propionate	Aerobic feast Aerobic famine	0.6 0.6	1 1	10 5	0.5 0.5	35 35	8.3-8.7 8.3-8.7	- -	(Patel et al., 2009)
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., 2017)
Municipal wastewater	Anoxic feast Aerobic famine	-	-	17	-	10-23	-	-	(Bengsston et al., 2017)
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)
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)

Continuation of **Table 2.3**.

Feedstock	Enrichment	OLR gCOD/(L d)	HRT d	SRT d	Cycle lenght d	Temperature °C	pH	Dominant Microorganism	Reference
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; Tamis et al., 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 T, 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 WAS (Cydzik-Kwiatkowska et al., 2016). However only a limited focus has been given in different studies on the abundance of the PHA production phenotype in WAS 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 (Bengsston et al., 2017). 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 *direct 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; Tamis et al., 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 (Moretto et al., 2020a; Moretto et al., 2020b; Valentino et al., 2018; Valentino et al., 2020; Valentino et al., 2019a; Valentino et al., 2019b).

Using the *direct accumulation* approach, accumulation up to 0.52 gPHA/gVSS were reported when feeding fermented waste VFA-rich streams to WAS from selected municipal and/or industrial WWTPs (Bengsston et al., 2017; 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 WAS 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 (Bengsston et al., 2017). The WAS from the pilot system was found to accumulate up to 0.49 gPHA/gVSS compared to the WAS 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.

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, both 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 co-polymer composition and its distribution, average molecular weight (Mw) and its distribution, thermal properties, mechanical properties and impurities (Bengsston et al., 2017; Laycock et al., 2013). 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 physico-chemical 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-hydroxyvalerate) monomers. From different pilot studies, the reported HV fraction 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., 2011). 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 (Bengsston et al., 2017; 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 (Cal et al., 2019; Chan et al., 2017; Koyama et al., 1997; Laycock et al., 2013; 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 (M. Porter et al., 2011; Yu, 2009; Yu et al., 2005).

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

Feedstock	Enrichment	Biomass	$Y_{PHA/VFA}$ gPHA-COD/gVFA-COD	PHA Content gPHA/gVSS	HV %	Mw kDa	Effluent	Reference
Acetic acid Acetic/Propionic acid	Aerobic feast Aerobic famine	Same as enrichment	-	0.21 ±0.02 0.25 ±0.03	0 5.6	2200 2300	Yes	(Patel et al., 2009)
Fermented dairy wastewater	Aerobic feast Aerobic famine	Same as enrichment	0.21-0.26	0.39-0.43	-	-	-	(Chakravarty et al., 2010)
Fermented beet process wastewater	Aerobic feast Aerobic famine	Same as enrichment	-	0.60	-	-	No	(Anterrieu et al., 2014)
Municipal wastewater	Aerobic feast 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 Aerobic famine	Same as enrichment	0.30 ±0.04*	0.70-0.80	16	-	N,P	(Tamis et al., 2014a)
Primary sludge centrate Acetic acid	Anoxic feast Aerobic famine	Municipal activated sludge	0.25-0.37	0.27-0.38 0.33-0.39	0-30 -	500 -	Yes	(Morgan-Sagastume et al., 2015)
Tomato waste centrate Acetic acid	Anoxic feast Aerobic famine	Municipal activated sludge	0.30-0.39 0.34-0.53	0.34-0.45 0.19-0.49	42-49 0	-	Yes	(Bengtsson et al., 2017)
Synthetic mixture Fermented candy factory wastewater Primary sludge concentrate	Anoxic feast Aerobic famine	Municipal activated sludge	0.35-0.48 0.28-0.52 0.28-0.55	0.37-0.43 0.37-0.43 0.28-0.42	0-44	700-1500	-	(Bengsston et al., 2017)
Fermented paper mill wastewater	Aerobic feast Aerobic famine	Same as enrichment	0.68	0.65-0.76	25	-	No	(Tamis et al., 2018)
Fermented OFMSW Acetic acid	Aerobic feast Aerobic famine	Same as enrichment	0.43-0.57 0.61-0.64	0.39-0.52 0.37-0.42	7-13 0	-	Yes	(Valentino et al., 2018)
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 Fermented OFMSW and SAS mix II Acetic acid	Aerobic feast Aerobic famine	Same as enrichment	0.50 ± 0.04 0.44±0.03 0.67±0.05	0.43±0.01 0.46±0.05 0.40±0.02	10 13 0	-	-	(Valentino et al., 2019b)
Fermented OFMSW and SAS mix	Aerobic feast Aerobic famine	Same as enrichment	0.59±0.04	0.52±0.04	-	-	-	(Moretto et al., 2020b)

Continuation of **Table 2.4**.

Feedstock	Enrichment	Biomass	$Y_{PHA/VFA}$ gPHA-COD/gVFA-COD	PHA Content gPHA/gVSS	HV %	Mw kDa	Effluent	Reference
Municipal wastewater	Anaerobic feast Aerobic (Anoxic) famine	Municipal activated sludge	-	0.03-0.07	-	-	-	(Larriba et al., 2020)
Fermented cellulosic primary sludge liquid Acetic acid	Aerobic feast Anoxic famine	Same as enrichment	0.61±0.07 0.60±0.06	0.44±0.06 0.47±0.05	21-41 0	-	Yes	(Conca et al., 2020)
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 Acetic/Propionic acid Potato-starch factory effluent	Aerobic feast Aerobic famine	Same as enrichment	0.67±0.15 - 0.69±0.15	0.52±0.05 0.52-0.61 0.45±0.06	0.1±0.1 24/63 1.9±0.8	548±66 181/602 547±78	-	(Morgan-Sagastume et al., 2020)

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 (Bengsston et al., 2017). Even though the produced polymer with different Mw would have different 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 (Bengsston et al., 2017; Morgan-Sagastume et al., 2015; Morgan-Sagastume et al., 2014; Morgan-Sagastume et al., 2020; Patel et al., 2009). 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 (Astolfi et al., 2020; Riccardi et al., 2020; Werker 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 WAS 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

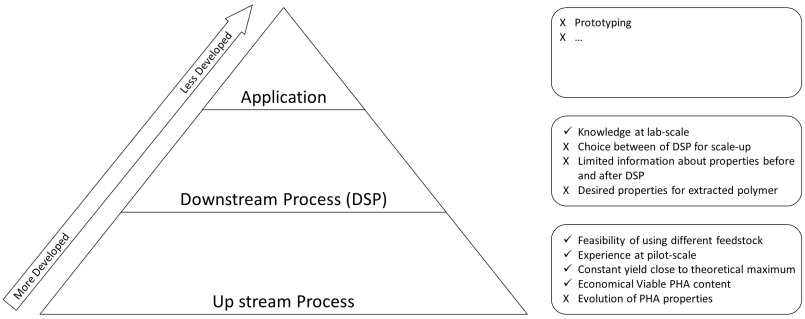

Figure 2.2: Summarized current development levels for PHA production process

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 (Burniol-Figols et al., 2020; Kosseva et al., 2018; Lorini et al., 2020). 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 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, regulatory 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 supply 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.

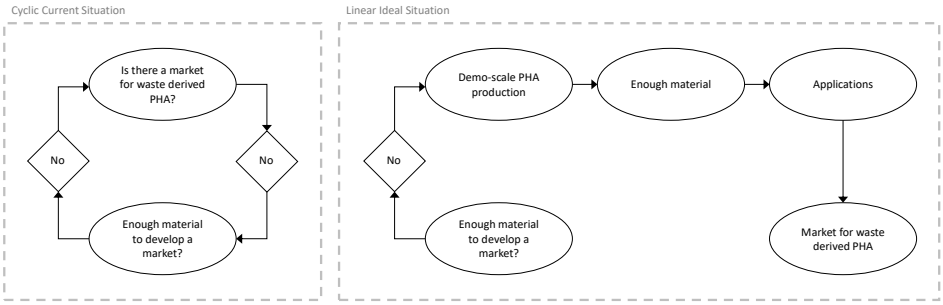

Figure 2.3: Depiction of dilemma in current scaling-up efforts of microbial community-based PHA production and how a demo-scale installation may contribute to support potential industrial implementation.

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 et al., 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 com-

mercially available pure culture derived PHAs in CRFs (Boyandin et al., 2017; Boyandin et al., 2016; Volova et al., 2016).

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 Bengsston et al. (2017). 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; Ferreira et al., 2020; Kale et al., 2007). Crystalinity, HV content and thermal stability are essential factors for the biodegradation of the polymer (Ferreira et al., 2020; Zaheer et al., 2018). Crystalinity affects the accessibility of the PHA degradation enzymes (Zaheer et al., 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 (M. Porter et al., 2011; Yu, 2009; Yu et al., 2005). 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 et al., 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:

- Focus on factors underlying ambiguities in observed differences in the performance of selection and understanding the evolution of polymer properties in the PHA accumulation process;
- 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;
- 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;

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, 2020b), 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.

Acknowledgments

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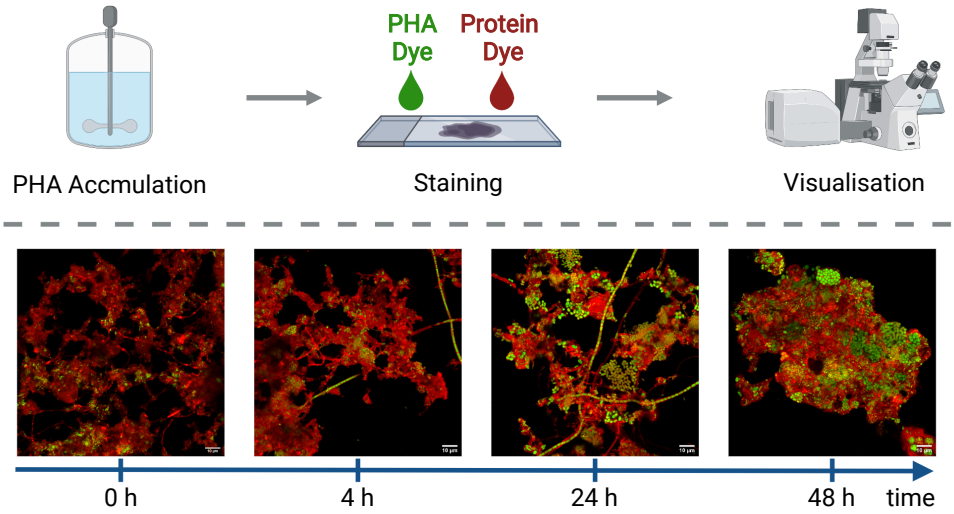
3

Visualisation of Polyhydroxyalkanoate Accumulated in Waste Activated Sludge



A modified version of this chapter has been submitted.

Highlights:

- *Selective staining enables visualisation of PHA accumulation development in biomass*
- *Only a fraction of the activated sludge was active in PHA accumulation*
- *PHA accumulation activity was heterogeneously distributed within and between flocs*
- *Two characteristic groups of PHA-storing bacteria were observed*
- *The two groups were distinguished in terms of flexible or stiff apparent cell wall*

Synopsis: *Activated sludge could accumulate PHA but different biomass PHA contents were found. In this work, staining methods to visualise PHA granule distribution and specific biomass components (DNA, RNA and protein) were developed, optimised and applied for a case study.*

Abstract
Polyhydroxyalkanoates (PHA) can be produced with waste activated sludge (WAS) from biological wastewater treatment processes. Distribution of PHA storage capacity in the activated sludge microbial community was evaluated during the accumulation process by methods of selective fluorescent staining with confocal laser scanning microscopy (CLSM). Staining methods to visualise PHA granule distribution alongside other specific biomass components (DNA, RNA and protein) were developed and optimised. In a case study, the selective staining was applied to a specific activated sludge used for PHA accumulation. PHA accumulation was performed at pilot scale in replicate experiments by controlled feeding of volatile fatty acids to reach biomass PHA contents of up to 0.48 gPHA/gVSS. *The PHA accumulation was visualised using the staining methods. For this activated sludge, not all but still a significant fraction of the biomass was engaged with the PHA storage activity. Accumulated PHA granules were heterogeneously distributed within and between flocs in the biomass. These observations suggested that the PHA content for the bacteria storing PHA was significantly higher than the average PHA content measured for the biomass as a whole.*

Keywords: *polyhydroxyalkanoate (PHA), bioplastic, activated sludge, staining, visualisation.*

3.1. Introduction

Polyhydroxyalkanoates (PHA) are a family of polyesters produced by a broad range of microorganisms. They are stored as intracellular granules and provide intermediate reservoirs for supply of carbon and energy (Anderson et al., 1990; Dawes et al., 1973; Van Loosdrecht et al., 1997). Microorganisms that accumulate PHA do so typically when environmental conditions result in stresses, such as a lack of an essential nutrient while organic carbon is available in excess (Koller et al., 2015; Rodriguez Perez et al., 2018; Zinn et al., 2001). The intracellular PHA granules are referred to as carbonosomes, due to the associated integrated outer layer of phospholipids with several specific polymer related functional proteins (Majone et al., 2014; Prieto et al., 2016). PHAs can be recovered from PHA-rich biomass using solvent extraction or water based purification methods (Kourmentza et al., 2017; Werker et al., 2020). The recovered purified semicrystalline polymers exhibit thermoplastic and mechanical properties similar to petroleum-based polyesters commonly used today (G.-Q. Chen, 2009; Pratt et al., 2019). Distinct from petroleum-derived polymers, PHAs are bio-based and biodegradable. Thus, research and development efforts advance to realize the potential for PHAs as an important recoverable resource by using wastewater as feedstock and microbial community based production methods (Á. Estévez-Alonso et al., 2021a; Werker et al., 2020).

Microbial community-based PHA production methods may be categorised as *enrichment accumulation* or *direct accumulation* depending on the source of the biomass (Á. Estévez-Alonso et al., 2021a). *Enrichment accumulation* starts with specifically cultivating a biomass. A feast-famine selection pressure is typically applied to produce a bacterial biomass using volatile fatty acid (VFA) rich feedstocks to enrich for PHA storing bacteria (Johnson et al., 2009; Kourmentza et al., 2017; Paul et al., 2020). The surplus enriched functional biomass is then fed with VFA rich substrates in a separate PHA accumulation bioprocess to reach its maximum PHA content.

Alternatively, *direct accumulation* exploits activated sludge from municipal and industrial biological wastewater treatment plants (WWTPs). In a PHA accumulation process, the waste activated sludge without further enrichment is directly fed with a VFA rich feedstock to reach its maximum PHA content (Bengsston et al., 2017). Wastewater activated sludge has exhibited significant PHA accumulating potential (Anterrieu et al., 2014; Bengtsson et al., 2017; Conca et al., 2020; Larriba et al., 2020; Morgan-Sagastume et al., 2015; Morgan-Sagastume et al., 2014). This significant level of PHA accumulating potential in activated sludge can arise because the environmental conditions in the WWTP are inherently, or purposefully engineered to be selective to favour survival for PHA storing phenotypes

(Van Loosdrecht et al., 1997). The selectivity can be due to a feast famine effect caused by the plug flow of the wastewater treatment lanes or day and night regime for the influent wastewater loading.

The PHA content in a dried biomass sample is readily quantified. Biomass PHA content is frequently reported as a part of accumulation performance assessments (Á. Estévez-Alonso et al., 2021a; Werker et al., 2020). *Enrichment accumulation* methods at laboratory scale have achieved a biomass PHA content of 90% gPHA/gVSS (Johnson et al., 2009). However, biomass PHA content under industrial conditions is often lower and can range between 40 and 80% gPHA/gVSS (Á. Estévez-Alonso et al., 2021a; Jiang et al., 2012; Tamis et al., 2014a). In these enrichment cultures, most of the biomass typically stores PHA. Thus, enrichment culture biomass is expected to have a high degree of enrichment for PHA accumulating microorganisms. As an example, *enrichment accumulation* was applied using the fermented organic fraction of municipal solid waste. A biomass PHA content of 46±5% gPHA/gVSS was achieved and staining revealed a very high degree of enrichment (Crognale et al., 2019; Valentino et al., 2019a). Therefore, the PHA accumulation potential, or maximum biomass PHA content reached for enrichment cultures with a high degree of enrichment (100%), estimates what the individual species of bacteria in the biomass can achieve on average.

With *direct accumulation*, surplus activated sludge has also resulted in biomass PHA content of about 50% gPHA/gVSS (Nikodinovic-Runic et al., 2013; Valentino et al., 2017). However, in these cases and in general in the research literature, biomass PHA contents are not reported together with information of the degree of enrichment. Determination of biomass PHA content alone is insufficient to evaluate both the inherent degree of enrichment for PHA accumulating microbes and average performance for individual cell accumulation potential. Lower or higher biomass PHA content for activated sludge can be due to lower or higher degree of enrichment or selected PHA-accumulating bacteria with a lower or higher maximal PHA content. To obtain a better insight, information on the distribution of the PHA storing phenotype, the degree of enrichment of activated sludge, is required.

Staining of PHA with counter-staining using different dyes can selectively visualise the intracellular PHA granules and their distributions in the biomass (Koller et al., 2015; Morgan-Sagastume, 2016). The most commonly applied dyes are Nile red, Nile blue A and Sudan black B (Koller et al., 2015; Morgan-Sagastume, 2016). Bright-field microscopy or a counter-stain for DNA via DAPI have also been applied in order to assess for the extent and distribution of polymer storage activity in the biomass. PHA granule staining can be

complemented furthermore with Fluorescence *In-situ* Hybridization (FISH) targeting 16S rRNA. FISH can reveal the consequent extent of microbial activity via a general bacterial probe EUB338 or can identify the abundance of specific microorganisms via specifically designed probes (Hugenholtz et al., 2002; Llobet-Brossa et al., 1998).

In the present study, complimentary counter staining methods were optimised specifically for municipal activated sludge biomass from a full-scale WWTP. The methods were developed using samples taken during 48-hour fed-batch PHA *direct accumulation* experiments at pilot scale. Different promising staining and confocal laser scanning microscopy (CLSM) strategies were initially screened. The best approaches were then optimised into standardised protocols. Staining methods included Nile blue A, Nile red, BODIPY 493/503 (BODIPY), DAPI, bright field, protein staining via SYPRO Red and FISH. Ultimately, optimal outcomes were developed by combining BODIPY and SYPRO Red, or BODIPY, FISH and DAPI. Systematic evaluations were then made in first application of the protocols that were developed, and these are reported herein.

3.2. Material and Methods

3.2.1. PHA Accumulation and Sample Fixation

PHA accumulation experiments were performed in a jacketed stainless steel 200 L pilot scale reactor with 167 L working volume. Influent volume flowed out as clarified effluent via a 16 L gravity settler and flow was actively recirculated between the main vessel and the settler. Temperature was maintained at 25 °C and mechanical mixing was constant at 230 rpm with a standard impeller. Aeration was via a membrane disk and constant at 50 L/min.

Waste activated sludge was from Bath WWTP (Rilland-Bath, the Netherlands) that treats for 470,000 person equivalents. The WWTP handles a mixture of municipal and industrial influent wastewater with screening and primary treatment. Secondary treatment is by tanks in series creating plug flow in a modified Ludzack-Ettinger activated sludge biological process (10 independent parallel treatment lanes with 20 days solids retention time). Phosphorus removal is by precipitation using $FeCl_3$. Previous studies have demonstrated consistent performance in PHA production by *direct accumulation* (Bengsston et al., 2017). Fresh grab samples of gravity belt thickened waste activated sludge (56.7 gTS/L) were delivered bi-weekly by courier on the same day. This source biomass was stored at 5 °C for no more than 2 weeks pending its use in accumulation experiments.

Prior to each accumulation experiment, the pilot reactor was first loaded with a determined weight of the thickened waste activated sludge that was then brought to a starting mixed liquor suspended solids concentration of about 2.5 gVSS/L by dilution with tap water. The reactor was brought up to 25 °C with constant mixing and aeration. Experiments were started after about 12 h hours once a steady state level of endogenous respiration was reached.

The feedstock was 20 gCOD/L acetic acid (VWR, the Netherlands) with added NH_4Cl and KH_2PO_4 (VWR, the Netherlands) for a COD:N:P (by weight) of 100:1:0.05. The pH was adjusted to 5.0±0.5 with NaOH (VWR, the Netherlands). Influent was given in discrete pulses of a fixed volume. The pulse volume targeted a peak substrate concentration of 100 mgCOD/L for each input.

Pulse input feedback control was by respiration level monitoring based on dissolved oxygen (DO) measurements (JUMO ecoLine O-DO, JUMO GmbH & Co. KG, Germany) as previously described by Werker et al. (2020). The process started with an acclimation step (Morgan-Sagastume et al., 2017). Acclimation comprised three feast-famine cycles. This meant that the first three pulse inputs were provided wherein DO was monitored to measure the (feast) time for the added substrate consumption. A famine time of 3 times the estimated feast time was then imposed before the next pulse input cycle. After acclimation, the accumulation process was automatically started. During accumulation feed pulses were given without any delay from one pulse to the next. Thus the accumulation process was characterized by feed-on-demand feast with a series of input pulses over 48 hours.

Over the course of each accumulation experiment, duplicate mixed liquor grab samples (50 mL) were taken at selected time points. One of the grab samples was acidified with addition of 98% H_2SO_4 (VWR, the Netherlands) to pH 2 and then centrifuged at 3248 RCF for 5 min at 4 °C (Beckman Coulter, CA, USA). The suspended solids pellet was separated from the supernatant and dried overnight at 105 °C. Dried pellets were ground and assessed by Thermal Gravimetric Analysis (TGA 2, Metller Toledo, Switzerland) for biomass PHA content (Chan et al., 2017). The duplicate grab samples were fixed directly with formaldehyde (Hugenholtz et al., 2002; Llobet-Brossa et al., 1998). 5 mL subsamples were combined with 5 mL of 1X Phosphate-buffered saline (PBS) (PanReac AppliChem, ITW Reagents, Spain) and 1.1 mL of 37% Formaldehyde (Sigma-Aldrich, the Netherlands). After incubation (~ 3 h or 12 h) at 4 °C, four rinse cycles were performed with 15 mL of 1X PBS by centrifugation (3248 RCF for 5 min at 4 °C), decantation, and resuspension. The pellet was then resuspended with 10 mL of 1X PBS and 10 mL of pure ethanol (VWR, the

Netherlands) and stored at 5 °C. After first analyses, fixed samples were preserved by storage at −20 °C for later possible use.

3.2.2. Staining Methods and Microscopy

3.2.2.1. PHA Staining and Biomass Counter-staining

Nile blue A staining was with 10 μL of fixed sample dispensed onto a glass slide followed by drying at 50 °C on a heating plate (van Loosdrecht et al., 2016). The dried solids were dipped into 1% (v/v) solution of aqueous Nile blue A and then incubated at 55 °C for 10 min. The slide was rinsed first with Milli-Q water (Merck, Germany) and then with 8% acetic acid (VWR, the Netherlands) for 1 min. The slide with stained sample was finally dried, then mounted with VECTASHIELD® HardSet™ Antifade Mounting Medium H-1400-10 (Vectashield) (Vector Laboratories, CA, USA), and sealed.

For Nile red staining, 1 mL of a fixed activated sludge sample was centrifuged at 12 000 RCF for 5 min. The supernatant was discarded and the pellet was resuspended with 1 mL of Milli-Q water. Nile red solution was added to reach a selected concentrations ranging between 1.6 μg/mL and 7.6 μg/mL. The sample was incubated at room temperature for 30 min. The incubated sample was centrifuged (12 000 RCF 5 min), and the harvested pellet was resuspended with 1 mL of Milli-Q water and mixing thoroughly. 10 μL of the stained and well-mixed sample was deposited to a clean glass slide, dried, mounted with Vectashield, and sealed.

BODIPY 493/503® (BODIPY) (Thermo Fisher Scientific, MA, USA) for PHA granule staining was combined with protein staining via SYPRO™ Red originally 5000X concentrated (Thermo Fisher Scientific, MA USA). Glass microscope slides with ten 5 mm diameter reaction wells were used (Paul Marienfeld GmbH & Co.KG, Germany). In each well, 5 μL fixed sample were dispensed followed by 0.5 μL of BODIPY with a concentration of 2 ng/μL. Then 5 μL of 100 times diluted SYPRO Red were added. The slide was then completely dried at 46 °C for at least 1 h. The prepared slide was finally washed with Milli-Q water, dried with the compressed air, mounted with Vectashield, and sealed. A negative control well was also included for each slide with the sample but with no dye applied.

3.2.2.2. Fluorescence *In Situ* Hybridization Combined with PHA Staining

Fluorescence *In Situ* Hybridization (FISH) was applied using EUB338-I with the sequence 5' GCT GCC TCC CGT AGG AGT 3' labelled with Cy5. FISH was combined with PHA staining via BODIPY and DNA staining via DAPI (Hugenholtz et al., 2002; Llobet-Brossa et al., 1998). Glass microscope slides with ten 5 mm diameter reaction wells were used again. 5 μL of fixed sample were loaded to each well, heat-fixed and then dehydrated with 50%, 80% and 100% ethanol. Then 10 μL hybridization buffer with 35% formamide was added followed by 0.5 μL of EUB338-I, BODIPY and DAPI with a concentration of 50 ng/μL, 2 ng/μL and 250 ng/μL, respectively.

The slide was incubated in a hybridization chamber at 46 °C for 1.5 h. The sides with hybridized sample were then washed and incubated in a pre-warmed buffer solution at 48 °C for 15 min. After subsequent washing with cold Milli-Q water, the slide was dried, mounted with Vectashield, and sealed. A negative control well was also included for each slide with the sample but with no dye applied.

3.2.2.3. Confocal Laser Scanning and Epifluorescence Microscopy

Bright field observations were made by a light microscope BX43 equipped with DP80 camera (Olympus, Japan) and by Confocal Laser Scanning Microscope LSM 880 (Carl Zeiss, Germany). For evaluation of selectively stained biomass, the LSM 880 with a Plan-Apochromat 63x/1.4 Oil DIC M27 objective (Carl Zeiss, Germany) was used. The stained sample areas were always surveyed first. Then, 11 fields of view with areas containing floc structures were selected randomly for a sequence of images. For each field of view, respective fluorescence dye emission signals were visualised at the optimum excitation wavelengths. Images from each excition wavelength were recorded into separate image channels with 16 scans averaged at a 16 bit depth.

Excitation wavelengths were: Diode 405-30 laser at 405 nm for DAPI, Argon laser at 488 nm for BODIPY, DPSS 561-10 laser at 561 nm for SYPRO Red and HeNe633 laser at 633 nm for Cy5. Imaging conditions, laser power and gain, were constant for the set of images from each reaction well. Composite images were generated by overlaying channels from the same field of view.

Similar laser power and gain levels were also used among the set of reaction wells for a given slide. The negative control wells were evaluated employing similar imaging

parameters to assess for any potential artefacts that could come due to auto fluorescence. Images were analysed using Fiji Image J (ImageJ2, Ver 1.52P).

3.3. Results and Discussion

The method development for the present investigation started with evaluation of staining methods for PHA-granules accumulated in waste activated sludge (Nile blue A, Nile red and BODIPY). After selection and tuning of BODIPY as the preferred stain, complimentary counter staining methods for other biomass components were established in order to visualize distribution of PHA with respect to biomass as indicated by protein, DNA, and RNA. A standard protocol was implemented and then applied to observe the development of PHA granule distribution in waste activated sludge based on replicated direct accumulation experiments. Qualitative observations were made for the distribution of PHA accumulation activity in the biomass with respect to the general distribution of biomass activity. Morphology of PHA storing communities, individual cells, and granules within cells were also assessed.

3.3.1. Selection of PHA Staining and Counter-staining Methods

For the visualisation of PHA granule distribution in the biomass, Nile blue A, Nile red and Sudan black B have been widely applied (Koller et al., 2015; Morgan-Sagastume, 2016). In this study, preliminary work was conducted with Nile blue A and Nile red using selected samples of PHA-rich biomass collected before and after 24 h of accumulation. No significant PHA staining signal was detected in the fresh waste activated sludge sample. After 24 hours of accumulation, Nile blue A stained for PHA (Figure 3.1). However, the signal from the stained PHA granules was found to be diffuse in definition and in this way the resolution was not ideal for evaluation of the PHA distribution in the biomass. Additionally, it was found that fluorescence signal was emitted in very broad laser excitation range from 405 nm to more than 600 nm. This excitation breadth meant that complimentary staining could not be applied without a risk for significant cross interference.

Nile red gave a better quality in the resolution for staining PHA granules compared to Nile blue A (Figure 3.1). This quality was in line with expectations based on the literature (Amirul et al., 2009). However, Nile red similarly exhibited a broad wavelength range for the dye excitation. Therefore, neither of the Nile dyes were found to be suitable for a strategy involving counter-staining and imaging for selected biomass components

in isolation from one and the other. An alternative PHA granule staining method was required.

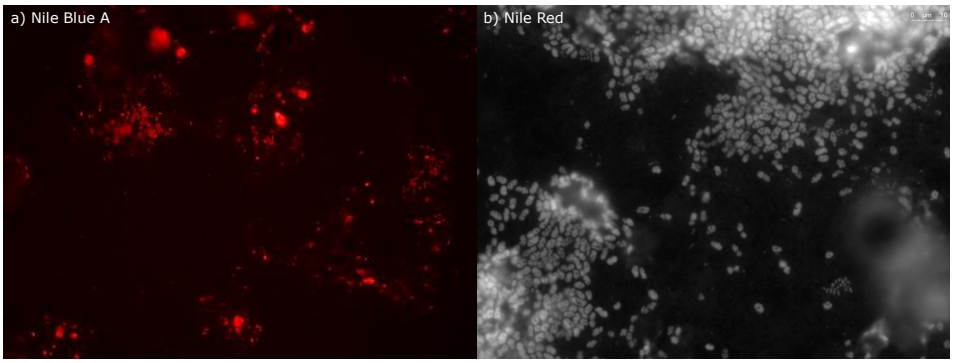


Figure 3.1: Visualisation of PHA accumulation in activated sludge after 24 hours by staining with Nile blue A (a) and Nile red (b).

Developments were directed to evaluate BODIPY 493/503, a lipophilic dye, which has also be applied to selectively stain for PHA granules (Koller et al., 2015). An optimal BODIPY PHA staining procedure for the PHA-rich municipal activated sludge was developed. Variations of sample incubation temperature, incubation time and applied BODIPY concentration were tested and compared based on the influence of the protocols on the fluorescence signal quality. Incubation trials were carried out at room temperature, 40 °C and 46 °C and with incubation times from 10 min up until all the liquid evaporated after about 1 h. It was found that maintained incubation at 46 °C until all the liquid evaporated yielded the best results in terms of the signal to noise ratio and the signal stability.

BODIPY concentrations in the range of 0.2 ng/μL to 20 ng/μL were evaluated. When the BODIPY concentrations were lower, the fluorescence signal was weak and bleached out during laser excitation within a few seconds. Rapid signal bleaching prevented to capture images with a better resolution by averaging multiple scans, to make a Z stacking composite images to reveal 3D structures, and to reuse the slides for repeated analyses. When the applied BODIPY concentration was too high, the image signal remained stable even for prolonged laser excitation. However, image quality in acuity suffered. This meant that finer details of the PHA granule distribution were not distinguishable. Excessive applied BODIPY loading also left unwanted dye residue after washing steps. This residue generated diffuse background signal which was responsible for the loss in image definition. A balance was struck at 2 ng/μL for enabling both good fluorescence signal stability, and resolution of details.

Excitation wavelengths of 405 nm, 488 nm, 561 nm and 633 nm were then examined. A strong fluorescence signal was obtained just at 488 nm. Thus, the other wavelengths could be used with other kinds of stains to reveal other biomass features. A narrow range of excitation and emission spectrum was essential to enable PHA granule staining in combination with the selective fluorescent staining of other biomass components.

BODIPY is a lipophilic dye. This makes such a stain not uniquely specific to PHA granules. Other lipophilic features of the biomass, the cell chemical structure, or other storage compound, such as intracellular lipid droplets, could be coincidentally stained (Cooper et al., 2010). The specificity of BODIPY towards PHA granules in the municipal waste activated sludge was examined. The biomass samples before and after the PHA accumulation experiments were stained with BODIPY. The same staining protocol was applied and images were acquired using similar imaging parameters. Some minor levels of point source fluorescence were observed in the biomass before accumulation Figure 3.2. This initial signal was similar to the experience with Nile blue A and Nile red. The observation fits also with initial measured PHA contents of around 0.01 gPHA/gVSS in the waste activated sludge. In contrast, the PHA specific signal after 24 h of PHA accumulation was intense and widely distributed throughout the biomass flocs. Thus, BODIPY resulted in the development of a staining signal corresponding directly to significant PHA accumulation. There were no signs of the stain illuminating other biomass generic or specific features such as cell membrane phospholipid structures, or floc extracellular polymeric substances.

Revealing distribution of PHA granules within the biomass morphological structures required a counter-stain. The counter-stain should show the compliment of the non-PHA biomass. Bright field or staining for specific cellular compounds could serve this goal. The latter was preferred due to an anticipated diversity in floc morphology and suspended solids chemical character for municipal activated sludge. Therefore methods were developed to evaluate accumulated PHA distribution with reference to specific, strategic, and stainable microbial cell chemical targets. Biomass protein and DNA were the selected targets to represent non-PHA biomass because of their ubiquitous presence, with contents of typically 55 wt% and 3.1 wt%, respectively (Madigan et al., 2015).

SYPRO Red was evaluated for generic cellular protein staining with dilutions from 1 to 5000 times of the original reagent (manufacture given as 5000X concentrated). An optimum balance of fluorescence stability and signal resolution was found at a 100 times dye dilution. Similarly, DAPI for DNA staining was optimal at 250 ng/μL. Non-PHA biomass staining was compared to floc structures as resolved using bright field microscopy. Figure 3.3 illustrates how both DNA and protein component staining were found to

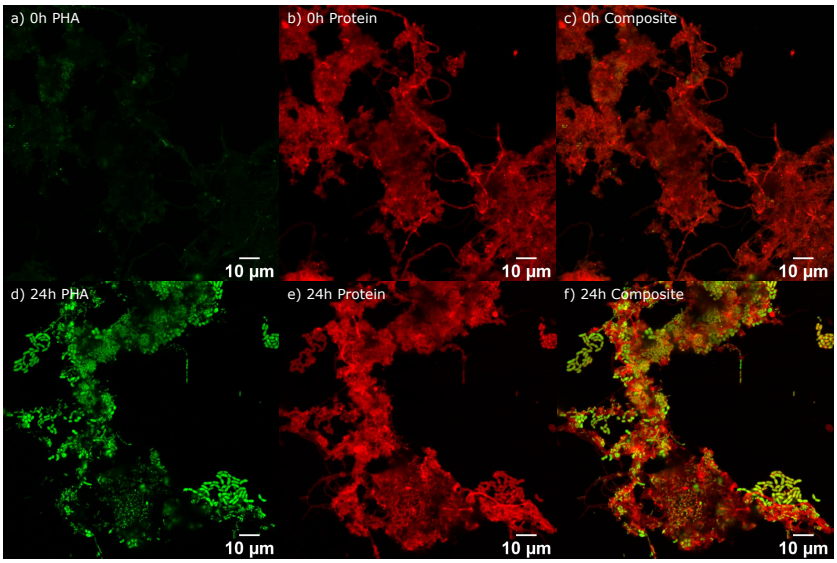


Figure 3.2: Visualisation of PHA accumulation in activated sludge by staining with BODIPY (PHA-green) and SYPRO (protein-Red). Samples at start of accumulation (top row) and after 24 hours of accumulation (bottom row).

overlap consistently with the bright field floc images. The consistently overlapping areas supported that DAPI and SYPRO Red staining could effectively provide a complimentary fluorescent signal from which to evaluate PHA distributions visualised by BODIPY. CLSM images from DAPI and SYPRO Red offered a degree of resolution and definition for individual cell morphology that was not accessible with the bright field images. This improved detail in resolution allowed for greater confidence in making morphological evaluations of the PHA storing organisms in the biomass.

Negative control wells for each slide with samples prepared without any added dye were evaluated using similarly applied parameters for image acquisition and post analysis. Negligible auto-fluorescence was observed with excitation wavelengths for BODIPY and SYPRO Red. Thus signal evaluations including pixel area counts were not biased by background signal noise. However, DAPI stain excitation at 405 nm did result in a low level of auto-fluorescence for the unstained biomass. DNA staining by DAPI was to be indicative of overall non-PHA biomass areas and distributions. A weak redundant auto-fluorescence under the stronger positive signal from DNA staining of biomass was therefore assumed to not introduce a bias for the purposes of the present investigation. Thus, the staining combinations with optimum conditions for BODIPY with SYPRO Red,

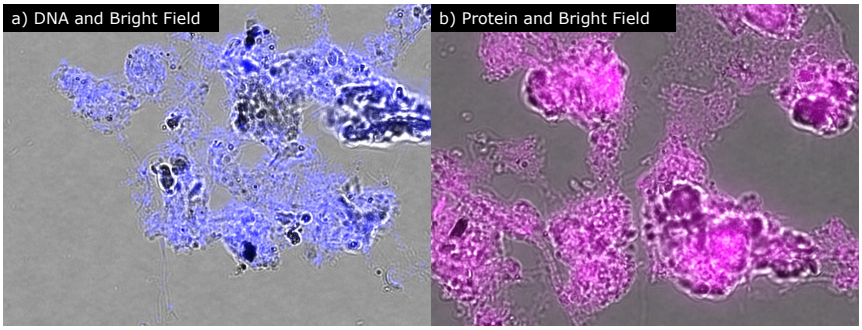


Figure 3.3: Bright field evaluation of the effectiveness of DNA (left in blue) and protein (right in pink) staining of activated sludge samples comparing with Bright field.

or BODIPY with DAPI, were applied further towards a routine protocol for staining and PHA distribution image analyses.

3.3.2. Application of Selective Staining in an Activated Sludge: PHA Distribution Development

As a case study, the developed selective staining method was applied to an activated sludge used directly for PHA accumulation. During the accumulation process, the biomass PHA content increased with pulse feeding of the acetic acid rich feedstock. A maximum plateau level of biomass PHA content was approached asymptotically by 27 h. Three replicate accumulation experiments gave similar plateau biomass PHA content levels of 0.48±0.02 gPHA/gVSS. The development of PHA distribution over time was followed also from samples fixed and stained using BODIPY and SYPRO Red. The process step of acclimation before accumulation has repeatedly been shown to increase the maximal accumulation potential for a waste activated sludge (Morgan-Sagastume et al., 2017). After acclimation some PHA storage could already be observed (Figure 3.4 and Figure 3.5). The initial PHA-granule distribution was not homogeneous. Patches of cell clusters with PHA were present after the third feast-famine cycle. Thus some microorganisms stored PHA with the applied acclimation feast, but not all of the polymer was metabolized in the time given during famine. In particular, free living long filamentous bacteria were noted to be the early accumulating organisms within the biomass. This morphological distinction could suggest for potential selective winners due to faster substrate up-take rate (Krishna et al., 1999). It could also suggest for differential rates of stored polymer metabolism during famine (Tamis et al., 2014b).

The pulse wise feeding rate was coupled to substrate uptake rate based on dissolved oxygen concentration as a proxy for the biomass oxygen uptake rate (Werker et al., 2020). The relative area of floc PHA granule coverage with respect to the non-stained biomass increased markedly over the course of the accumulation process. These observations were readily made with SYPRO Red counter staining. For example, Figure 3.4 shows typical outcomes during accumulation after 0 h and 48 h. However, even by 48 h, not all the biomass floc area became covered with stored PHA. Biomass in municipal activated sludge may have significant fractions of inert solids, non-PHA storing organisms, as well as dormant microorganisms. In dedicated PHA enrichment cultures virtually all bacterial cells are observed to accumulate PHA (Crognale et al., 2019; Tamis et al., 2014a). The degree of enrichment can be up to essentially 100 percent for enrichment accumulation biomass. This level of enrichment is not generally expected for municipal waste activated sludge.

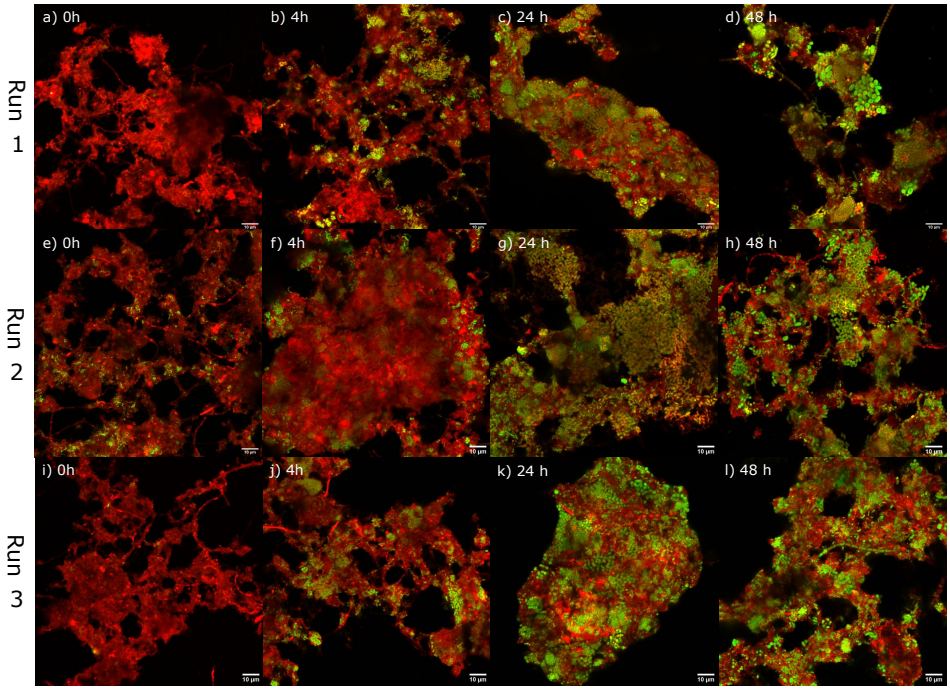


Figure 3.4: *Direct accumulation* replicate experiments with developments over 48 h revealed by combined PHA (green) and protein (red) staining.

PHA and protein biomass staining methods when combined helped to visualise cell morphology and the heterogeneous nature of PHA granule distribution during *direct*

accumulation. 16S rRNA based microbial activity measurements and phylogenetic identification based on specific FISH probes were also considered. The goal was to associate specific genotypes in the activated sludge with PHA production. Phylogenetic identification based on FISH into PHA and protein biomass staining was challenging due to fluorescent emission signal interference with either BODIPY or SYPRO Red signals excited at 488 nm and at 561 nm, respectively. A fluorophore in conjunction with a FISH probe was sought with an excitation wavelength in the UV range (405 nm). Thus, fluorophores including Alexa 405, Atto 425, Eterneon 393/523 and Pacific blue conjunct with EUB338 I at both at 3' and 5' ends were evaluated. Labeled probes were applied at concentrations in the range from 50 ng/L to 500 ng/L. Probe concentrations were tested in combination within ranges of hybridization times (1 h to overnight), hybridization temperatures (37 °C to 50 °C), and formamide concentrations (0% to 50%). Unfortunately, under all the tested combinations of conditions, low quantum yields for the dyes excited in the ultraviolet range resulted. The low yield limited the ability to generate sufficient fluorescence signal. The FISH signals were not sufficiently distinct with respect to an auto-fluorescence negative control using the same imaging parameters. As an alternative approach, a combination of PHA, RNA and DNA staining was evaluated.

The combination of PHA, RNA and DNA staining was performed using DAPI, EUB338 I and BODIPY. DNA with 16S rRNA counter staining was to reveal distribution of cellular viability with activity in the biomass with respect to zones in the distribution of PHA storing activity. Figure 3.5 illustrates how the PHA (green) and DNA (blue and purple) counter-staining approach gave similar outcomes to what was observed from PHA (green) and protein (red). DNA staining gave a less pronounced definition of individual bacterial cell boundaries compared to protein staining. However, the DNA staining could still be used to estimate the fractions of the biomass accumulating PHA.

16S rRNA was applied to indicate for distribution of overall microbial activity with respect to PHA accumulation activity. Distribution of RNA (purple in Figure 3.5) suggested that the active fraction of individual flocs was already significant even by the start of the accumulation process. By 48 h of *direct accumulation*, non-active biomass fraction could be discerned. These distributions for active and non-active floc fractions were seemingly without specific pattern or coupling specifically to PHA storage activity. No obvious spatial pattern of correspondence was observed. Thus an impression was that even if all PHA accumulating microorganisms exhibited 16S rRNA activity, this activity was not exclusive. Other fractions of biomass exhibited 16S rRNA activity that was not coincident with PHA storage (Purple in Figure 3.5).

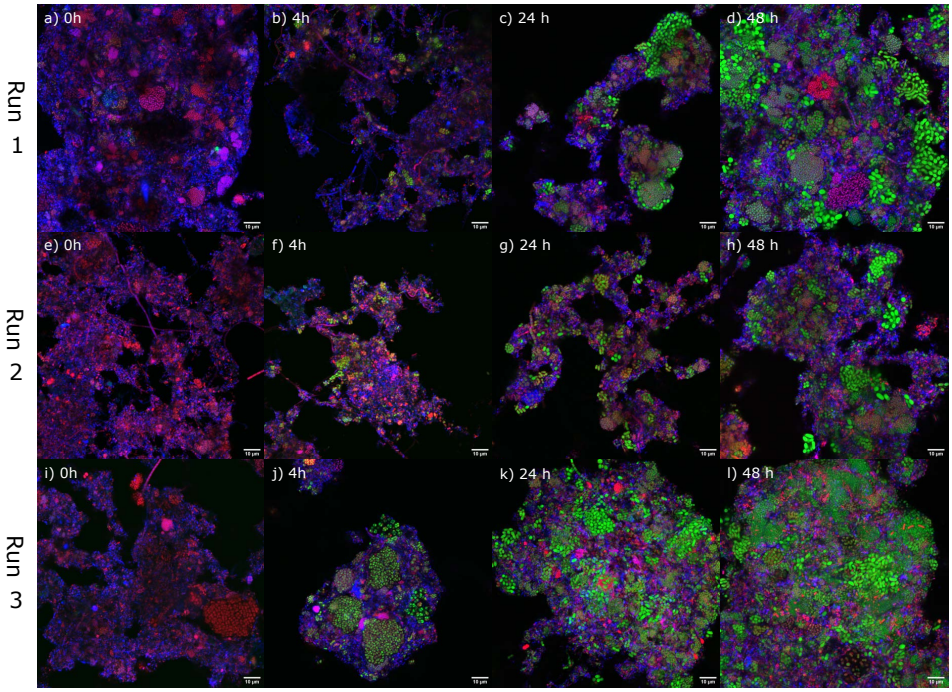


Figure 3.5: *Direct accumulation* replicate experiments with developments over 48 h revealed by combined PHA (green), DNA (blue), and 16S rRNA staining (red).

Microbial activity away from the distributed floc zones of PHA storage, and estimated average yield of PHA production on acetate (0.26±0.03 $gCOD_{PHA}/gCOD_{Acetate}$ at 48 h), are indicative for the presence of flanking microbial activity. A maximum yield due to PHA storage and no microbial growth is expected to be 0.75 $gCOD_{PHA}/gCOD_{Acetate}$ (Marang et al., 2013). The biomass PHA content level became nevertheless stable between 27 and 48 hours but substrate utilization efficiency for PHA production was low. Stable average biomass PHA content with ongoing microbial activity are indicative of PHA storage with active microbial growth (Valentino et al., 2015).

3.3.3. Application of Selective Staining in an Activated Sludge: Morphology of Microorganisms

The biomass used for PHA accumulation was a flocculating activated sludge. Different morphological structures in the flocs were observed using bright field microscopy

and CLSM during the *direct accumulation* experiments (Figure 3.4, Figure 3.5 and Figure 3.6). Similar patterns of morphological diversity were observed in the different batches of waste activated sludge that were used. These structures included round amorphous compact micro-colonies, irregular open structures abundant with filamentous microorganisms, low abundance of fingered zoogloaes, free-living filamentous and, also, planktonic cells (Figure 3.6). The majority of the flocs were found to be larger than 100 μm and with a thickness more than 100 μm. Larger flocs were seen to be bridged by filamentous bacteria, while the smaller flocs were not. The observed floc and cell morphologies were typical for municipal activated sludge (Tansel, 2018). The morphological characteristics did not change during direct accumulation (Figure 3.6). However, by 48 h, signs of growth became apparent with increased abundance of planktonic cells and cells that were loosely attached to the floc structures. Those newly appearing cells were with similar morphology and most of them were also accumulating PHA. These observations were indicative of active growth with both PHA storing and non-storing microorganisms later during the process.

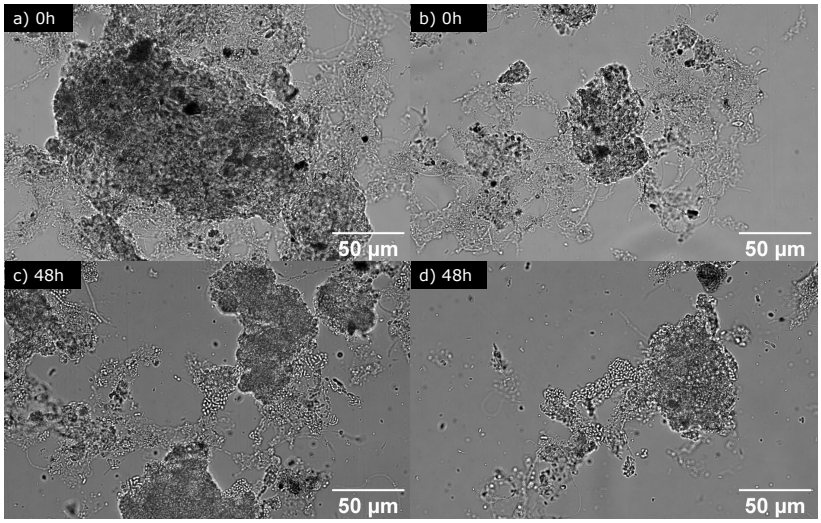


Figure 3.6: The bright field images of typically observed floc structures after 0 h and 48 h *direct accumulation.*

Protein and DNA stained images provided better visualisation of detail for distinguishing cell morphology than was possible from the bright field images (Figure 3.4 and Figure 3.5). By the time (27 h) when PHA content reached a plateau level of 0.48±0.02 gPHA/gVSS, two floc fractions became clearly delineated. PHA accumulating microorganisms were

bacterial cells where the stained PHA-granule structures overlapped with protein or DNA stained structures. Microbial cells without stained PHA-granule structures after 27 h could be interpreted as the non-PHA accumulating biomass fraction.

Interestingly, the level of discrimination for cellular details evolved and improved with PHA storage (Figure 3.4 and Figure 3.5). Specifically, individual cells became more readily discernible, and segmentation between cells in clusters became more clearly visible. Different levels of heterogeneity were observed between individual flocs and individual microbial cells. Clusters of populations of PHA accumulating microorganisms were observed to be distributed heterogeneously among different flocs. Figure 3.7 shows illustrative examples of thin and thick filaments, cocci, rod-shape and short-rod shaped microorganisms that were observed to be accumulating PHA. Filamentous morphotypes that have been reported typically in activated sludge could not be easily classified especially before any PHA accumulation. Different types of thick filaments that are typical to municipal activated sludge were found to accumulating PHA. Those filaments were mainly smoothly curved within the floc structures, or free-living but bridging the flocs. Square and rectangular individual cells were the dominant shape in these cases. Barrel shaped filaments were found less frequently. For all thick filaments, after 48 h *direct accumulation*, the cross walls between cells became clearly visible.

Classification, e.g. by using specific probes providing morphotype identification could be applied for added benefit in future work. Such probes would allow for further characterisation and especially for following changes of the morphology before and after the PHA accumulation. Non-filamentous PHA accumulating microorganisms sharing similar morphology were observed as distinct dense micro-colonies. These islands of compact micro-colonies represented the dominant observed fraction of the PHA accumulators distributed in the biomass. The clustered morphotype suggested that dominant PHA accumulating species or families might exist in the municipal activated sludge. However, assessment of dominant PHA accumulating families or species needs to be further evaluated by means of molecular phylogenetic measurements. Specific FISH probes could be applied but this step was beyond the scope of the present investigation focused on the counter staining method development and PHA distribution visualisation.

The degree of heterogeneity in distribution and the pockets of more intense activity for PHA storage were unexpected. A significant distinct fraction of the biomass fraction was observed that was not accumulating PHA. Measurements of average dried solids biomass PHA content will not reveal this heterogeneity because the analyzed sample size is too large. Since two significant distinct biomass fractions were observed, the average biomass

PHA content in the PHA accumulating fraction is expected to be significantly higher. If the fractions are separable then, a biomass with much higher PHA content could be harvested as part of the downstream processing for the polymer recovery.

Image resolution was sufficient to estimate size of the PHA accumulating bacteria. Thin filamentous and coccus like microorganisms with a diameter smaller than 0.5 μm did not evolve with obvious size changes due to stored polymer. However, sizes of other PHA accumulating microorganisms did increase, especially in length and width of rod shape microorganisms, and width of the thick filaments. The size of the rod shape microorganisms were between 0.5 and 1.0 μm or smaller before the accumulation. The size of those microorganisms increased to about 2 to 3 μm with granules. PHA granules caused distortions to the morphotypes that made native rod or coccus organisms, with dimensions smaller than 2 μm, and typically around 0.5 μm, become morphologically indistinguishable. The length of filaments could become longer and more than 100 μm. The width of the thick filaments doubled from about 0.5 μm to 1 μm for the square and rectangular types. The barrel shaped filaments had a width of around 2 μm after 48 h.

However, change in cell size and morphology was not evident for all types of cells in the PHA accumulating fraction of the biomass. Two distinct groups of PHA accumulating microorganisms were observed for this specific activated sludge. In one group, cell sizes increased significantly due to PHA storage, while in the other they remained the same. The first group exhibited an adaptive stretchability. This expandability suggested reduced cell wall stiffness. The other group were more rigid and thus maintained an apparently higher degree of cell wall stiffness. Rigid cells are reported to express a lower capacity to store PHA (Shen et al., 2019; Zhang et al., 2018). Thus the biomass PHA content in the PHA storing fraction may be bimodal with rigid cell types having lower accumulation potential. Heterogeneity of biomass rigidity also can influence considerations important for the polymer recovery. During the post accumulation downstream processing stretched (stressed) cells may be more susceptible to lysis due to disturbances (shear forces or chemical pretreatments). Polymer may be easier to recover, from the stressed distended biomass fraction, but also easier to loose in the process. The opposite may be expected when recovering polymer stored from rigid cell structures. Rigid cells may not lyse so readily and purification of the polymer through washing steps may be less effective. PHA recovery and purification methods after direct accumulation will need to address the challenge for all cell types in order to reach optimal outcomes for polymer yield and quality.

The distribution of RNA and DNA also was influenced by PHA granule storage. Before

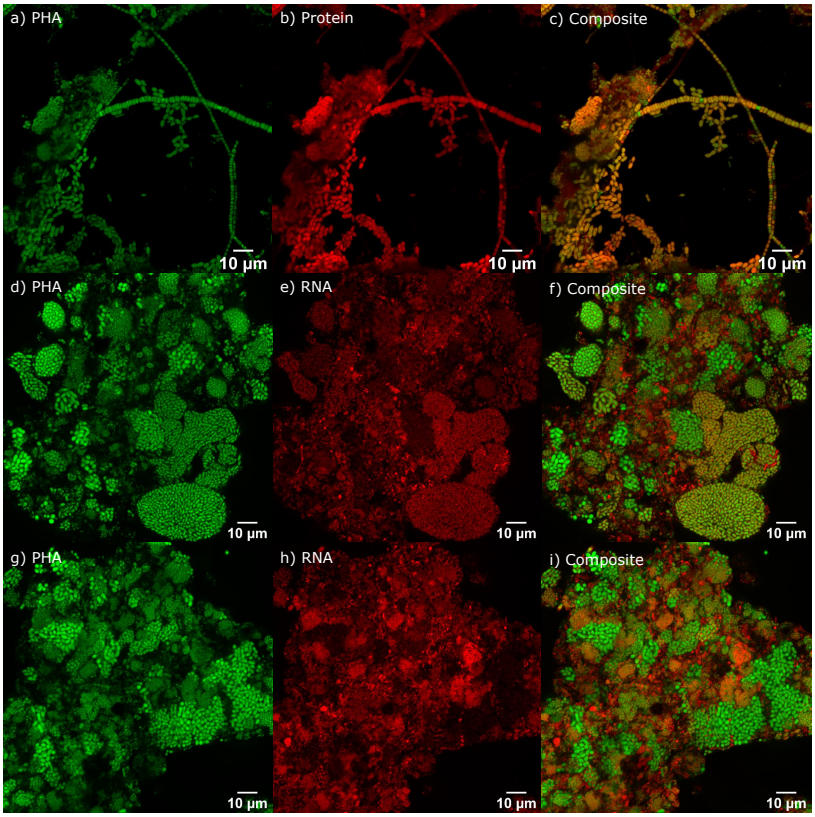


Figure 3.7: Morphology of PHA accumulating microorganisms after 27 h *direct accumulation* with PHA biomass content at the saturation level of about 0.48±0.02 gPHA/gVSS.

direct accumulation, RNA and DNA were more evenly distributed in the cell (Figure 3.8 (f)). However, after 48 h accumulation, DNA and RNA were observed to be displaced and crowded out within the cytoplasm due to the granules (Figure 3.8 (j, k,l)). Before PHA accumulation, RNA was distributed uniformly over the whole cell. After PHA accumulation, RNA was observed to distribute like a ring surrounding the PHA granules. It was found the DNA of the cells were pushed even further to a different focus plane compared to the RNA. Thus, where there was PHA accumulated Figure 3.8 (g, k) there was no DNA signal (Figure 3.8 (i, k)) in the same area. PHA storing microorganisms are known to be able to store PHA concurrent to cell division (Jendrossek et al., 2014). It is interesting to consider further how or if this change in the distribution of vital cellular machinery influences function.

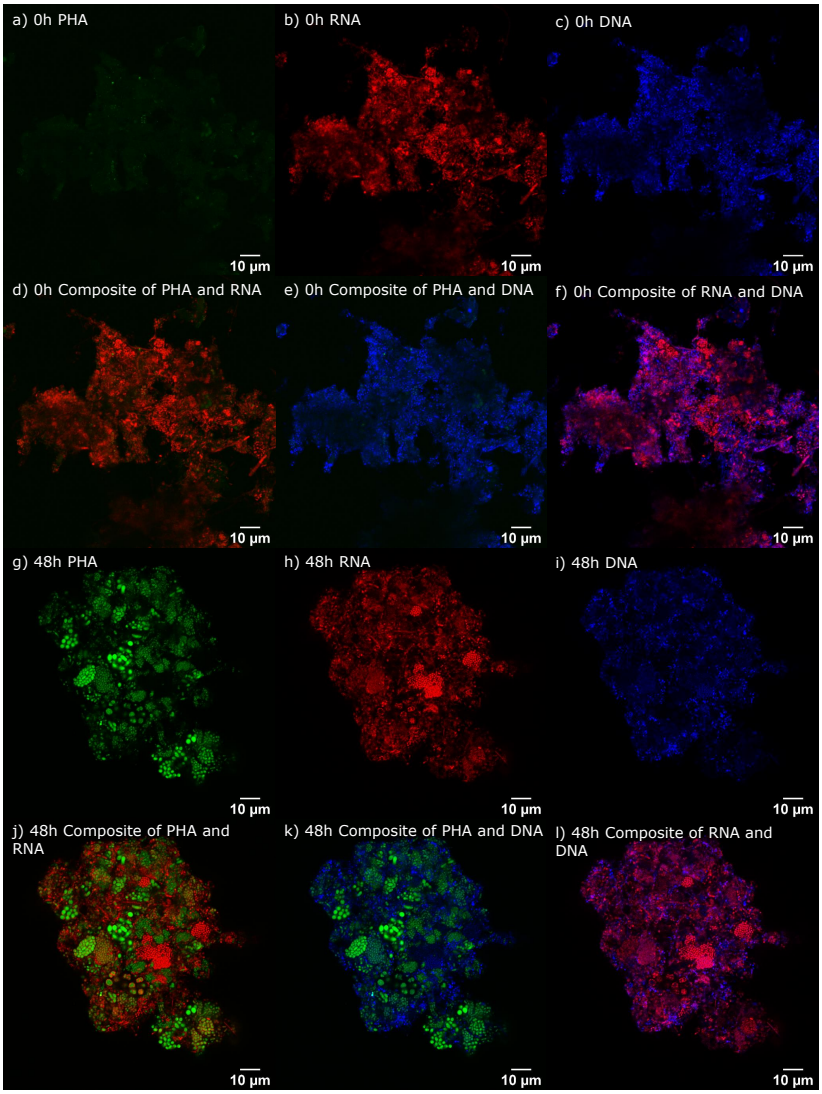


Figure 3.8: Distributions of PHA, RNA, DNA and the composites initially and after 48 h *direct accumulation* experiments.

Non-PHA accumulating microorganisms were thin filaments and rod shaped cells. The rod-shaped microorganisms without PHA-granules were not considered to be unique or distinct in morphology to the rod-shaped PHA accumulating microorganisms. It was also found that some of the non-PHA accumulating microorganisms also formed clusters and could be characterized to be forming less compact flocs. Organic substrate supply with limiting nutrients can promote for PHA storage as well as excess extracellular polymeric substances (EPS) production (Ajao, 2020). In the presence of excess of organic carbon, there are two distinct responses which are PHA accumulation or EPS production. The EPS formation would lower the yield of PHA on substrate. In continued work, it would be of value to explore ecophysiology roles, or competitive strategy, of those non-PHA accumulating microorganisms during *direct accumulation*. If that activity can be mitigated, then volumetric productivity can be increased significantly. Alternatively, the understanding can be directed to augmenting selection pressure during the wastewater treatment. Increased selection pressure would reduce the fraction and activity of the non-storing biomass in *direct accumulation*.

3.3.4. Application of Selective Staining in an Activated Sludge: Distribution of PHA Granules within Individuals Cells

Selective staining in combination with CSLM enabled resolution of details to discriminate between floc morphologies. In some cases, resolution was to the level of detail of PHA granule morphology. Section 3.3.3, reported how similar morphotypes of PHA accumulating microorganisms were found to be clustered. The fluorescent emission of the stained PHA-granule covered contiguous floc areas. An impression from replicate experiments was that similar morphotypes progressed similarly in build-up of stored PHA content. It was also observed that different clusters of morphotypes accumulated distinctly different estimated size and numbers of the PHA granules per cell. Seven different types of PHA granule morphologies were identified as illustrated by Figure 3.10. In some cases, individual PHA granules could be distinguished quite clearly. This level of detail that typically requires Transmission Electron Microscopy suggests a high degree of selectivity for PHA staining using BODIPY. For cocci and rod shaped bacteria, smaller cells were seen to store smaller individual PHA granules as also found for pure culture rod-shaped bacteria like *Ralstonia eutropha*, *Cupriavidus necator* (Zhang et al., 2018). Observations for PHA granules in filaments were similar to those reported by Dionisi et al. (2002). The number of granules per cell varied and was estimated to be from 3 to more

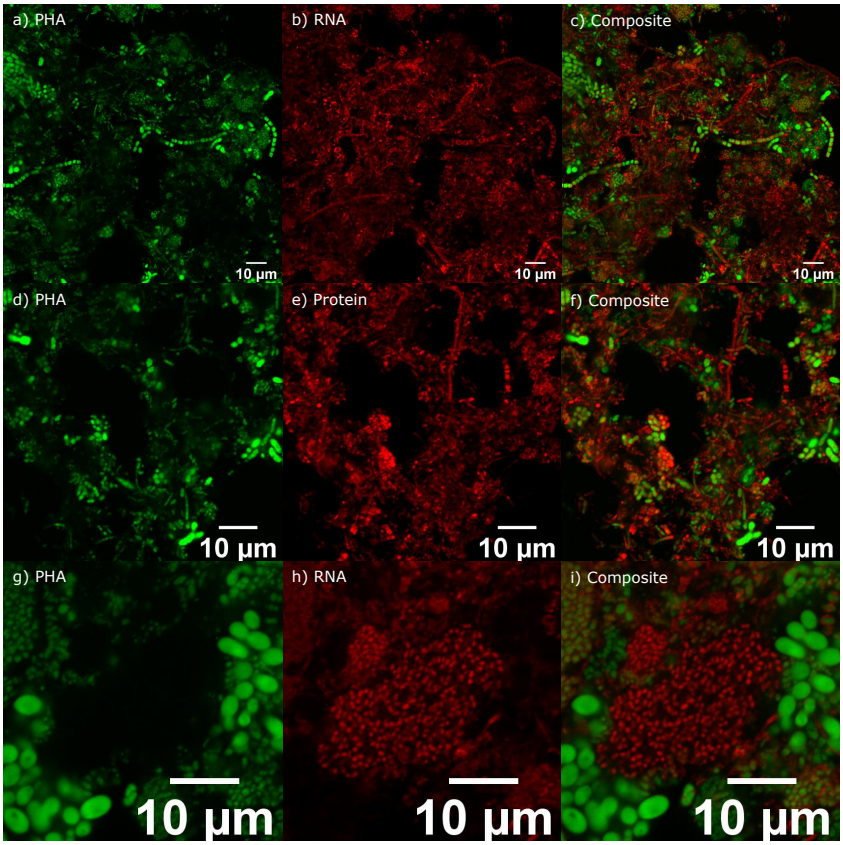


Figure 3.9: Morphology of PHA accumulating (green overly with red) and non-PHA accumulating microorganisms (only green) from *direct accumulation* experiments.

than 10 as is typical for pure cultures (Zhang et al., 2018). Ultimately, size and number of PHA granules can become limited due to cell size Shen et al. (2019).

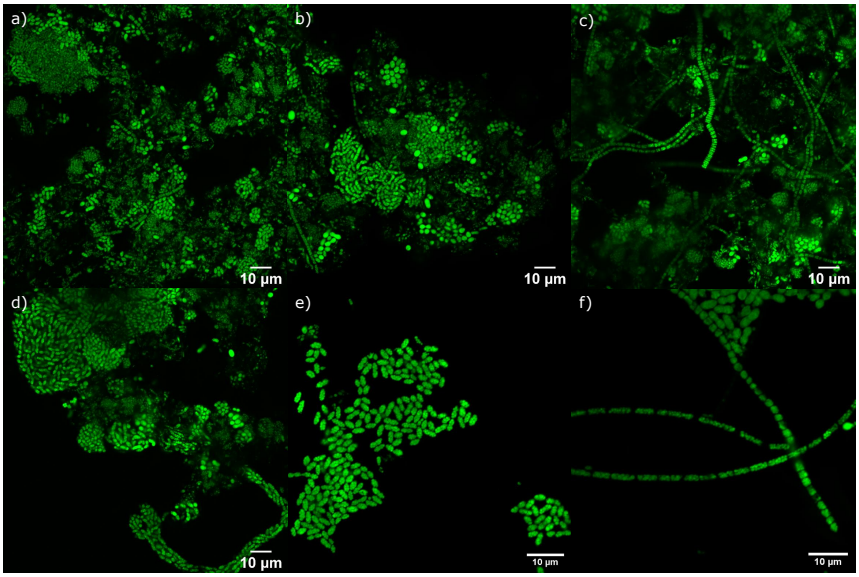


Figure 3.10: Morphology differences for PHA granules stored in the waste activated sludge from replicate *direct accumulation* experiments.

Acknowledgement

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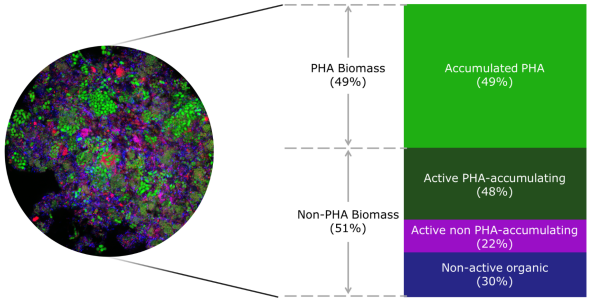
4

Quantification of Polyhydroxyalkanoate Accumulated in Waste Activated Sludge



[1] This chapter has been published: Pei, R., Vicente-Venegas, G., Van Loosdrecht, M. C., Kleerebezem, R., & Werker, A. (2022c). Quantification of Polyhydroxyalkanoate Accumulated in Waste Activated Sludge. *Water Research*, *221*, 118795. https://doi.org/10.1016/j.watres.2022.118795

Highlights:

- *Staining microscopy and image analysis can quantify PHA distribution in biomass*
- *Volume and mass ratios of stored PHA to non-PHA biomass were correlated*
- *Degree of enrichment for a PHA storing activated sludge (55%) was estimated*
- *Distribution of 16S rRNA levels was not specific to PHA accumulation activity*

Abstract
Polyhydroxyalkanoate accumulation experiments at pilot scale were performed with fullscale municipal waste activated sludge. Development of biomass PHA content was quantified by thermogravimetric analysis. Over 48 hours the biomass reached up to 0.49 ± 0.03 gPHA/gVSS *(n=4). Samples were processed in parallel to characterise the distribution of PHA in the biomass. Selective staining methods and image analysis were performed by Confocal Laser Scanning Microscopy. The image analysis indicated that nominally 55% of this waste activated sludge was engaged in PHA storage activity. Thus even if the biomass PHA content reached* 0.49 gPHA/gVSS, *the accumulating fraction of the biomass was estimated to have attained about* 0.60 gPHA/gVSS. *The combination of quantitative microscopy and polymer mass assessment enabled to distinguish the effect of level of enrichment in PHA storing bacteria and the average PHA storage capacity of the accumulating bacteria. The distribution of microbial 16S rRNA levels did not follow a measurable trend during PHA accumulation.*

Keywords: *polyhydroxyalkanoate (PHA), biopolymer, activated sludge, staining, image analysis, Thermogravimetric analysis (TGA).*

4.1. Introduction

Polyhydroxyalkanoates (PHA) are polyesters that can be accumulated as intracellular granules by many types of microorganisms especially under dynamic environmental conditions (Anderson et al., 1990; Dawes et al., 1973; Van Loosdrecht et al., 1997). Typically, these dynamic conditions are created by alternating the presence and absence of organic carbon sources and/or electron acceptors. Under dynamic conditions, PHA accumulation is a competition strategy for microorganisms to thrive (Majone et al., 1999; Reis et al., 2003; Van Loosdrecht et al., 1997). The accumulated intracellular polymers can be harvested and applied as biobased and biodegradable polyesters. PHAs are functional and suitable in many kinds of applications compared to fossil oil derived polyesters used ubiquitously today (Laycock et al., 2013; Pratt et al., 2019).

Microbial community-based PHA production processes exploit purposefully imposed or inherently present environmental conditions in open microbiological processes that favour the growth and enrichment of PHA storing phenotypes (Á. Estévez-Alonso et al., 2021a; Kleerebezem et al., 2007; Kourmentza et al., 2017). Depending on the source of the PHA-storing biomass, microbial community-based PHA production methods may be denoted as either *enrichment accumulation* or *direct accumulation* approaches. *Enrichment accumulation* refers to PHA production with a biomass that has been produced purposefully with optimised selection conditions. A majority of the selected bacteria in the biomass are expected to produce PHA. A *direct accumulation* process uses waste activated sludge produced in municipal and/or industrial biological wastewater treatment plants (WWTPs) (Bengsston et al., 2017; Morgan-Sagastume, 2016). Notwithstanding that the principal function of the process is to treat wastewater, selection of PHA-storing microorganisms will occur due to the inherent dynamic process conditions (Van Loosdrecht et al., 1997). The selection could be due to the cyclic day-night loading regime in wastewater supply and/or a plug flow character in the wastewater treatment process (Werker et al., 2020). The wastewater treatment processes select for a microbial community that has broad functional diversity. As a result, there will be non-PHA storing microorganisms in the *direct accumulation* biomass essential for the treatment of influent contaminants (Pei et al., 2022b).

PHA production is accomplished in a side-stream fed-batch bioprocess where the supplied PHA-storing biomass is fed with volatile fatty acid rich substrates to reach the maximum biomass PHA content. The volatile fatty acid rich substrates can be derived for example from the fermentation of wastewater, organic fraction of municipal solid waste or the primary sludge of WWTP (Á. Estévez-Alonso et al., 2021a). The use of fermented

primary sludge also offers the possibility of converting the WWTP into PHA production facility without modifying the main treatment process. Over the past 10 years, different pilot scale studies demonstrated the technical feasibility of microbial community-based PHA production processes using different feedstocks (Á. Estévez-Alonso et al., 2021a).

In the PHA production process, maximum biomass PHA content is a critical performance factor that influences the economic viability of commercial advancements (Á. Estévez-Alonso et al., 2021a; Paul et al., 2020; Valentino et al., 2017). Consequently, in almost all the microbial community-based research literature to date, maximum achievable biomass PHA content has been a key parameter when assessing the performance of the PHA production process. Biomass PHA content is typically quantified by the mass of PHA accumulated with respect to the total volatile suspended solids (VSS). Active biomass is commonly approximated by the VSS minus the PHA mass in these solids. Microbial community-based approaches have been reported to have a maximum biomass PHA content between 30 and 90% gPHA/gVSS (Á. Estévez-Alonso et al., 2021a). Implicitly it is often assumed that improvement in PHA production is reflected by improvement in selection and mitigating flanking populations during accumulation (Tamis et al., 2014a).

Observed different biomass PHA content for microbial community-based approaches may be attributed to two main factors. One factor is the fraction of PHA-storing bacteria in the biomass defined as a degree of enrichment. The other factor is the PHA accumulation capacity of the actively PHA-storing bacteria. The current methods for measurement of biomass PHA content do not provide insight to distinguish between these two factors. To assess the individual cellular PHA distribution, flow cytometry or Raman spectroscopy may be applied. In pure cultures, Nile red or BODIPY staining has been employed with flow cytometry to quantify the PHA content and to study the PHA content in single cells (Kacmar et al., 2006; Saranya et al., 2012; Vidal-Mas et al., 2001). Similarly, methods have been explored based on floc disruption and statistics of cell-to-cell PHA content by means of Raman spectroscopy of the dispersed microbial biomass (Majed et al., 2009). In microbial community-based PHA research, these methods are less applicable due to problems of dispersion of the biomass in single cells and the variation of cellular morphologies in microbial communities. Moreover destruction of the floc structure obscures to obtain information from the distribution of the PHA granules and PHA-storing bacteria in the communities.

The aim of the present work is to develop the selective staining methods for a quantitative estimation of the municipal activated sludge degree of enrichment and, consequently, the average PHA accumulation capacity of the PHA storing fraction of that biomass. In

the present work, the PHA-rich biomass was produced with a municipal activated sludge using the *direct accumulation* approach at pilot scale. The progress of polymer accumulation was monitored over time with grab samples for biomass PHA content. Recently developed selective staining for visualisation on parallel samples was also applied (Pei et al., 2022b). Obtained Confocal Laser Scanning Microscopy (CLSM) images were analysed to quantify the PHA distribution.

4.2. Material and Methods

4.2.1. PHA Accumulation Methods and Sampling

A well-mixed 200 L jacketed stainless steel reactor with 167 L working volume for PHA accumulation was operated as described previously (Pei et al., 2022b). The accumulation feedstock was acetic acid (20 gCOD/L) with added NH_4Cl and KH_2PO_4 (VWR, the Netherlands)to a COD:N:P of 100:1:0.05 (by weight). The feedstock was adjusted to pH 5.0±0.5 with NaOH pellets (VWR, the Netherlands). The substrate was supplied in a fed-batch feed-on-demand process controlled according to biomass respiration response monitored (JUMO ecoLine O-DO, JUMO GmbH & Co. KG, Germany) by dissolved oxygen trend as described in Werker et al. (2020). The source biomass that was evaluated for the purposes of the this study was gravity belt thickened (nominally 5.5 percent total solids) waste activated sludge from WWTP Bath, the Netherlands.

Thickened activated sludge was diluted with tap water to target 2.5 gVSS/L and brought to operating temperature (25 °C) in the active working volume. Aeration and mixing were up to 12 h until steady state dissolved oxygen levels indicated a steady biomass endogenous respiration level. This pre-aeration was followed by acclimation applied as a sequence of three feast-famine cycles (Morgan-Sagastume et al., 2017). At the beginning of the feast period, a pulse of substrate targeted a reactor acetic acid concentration of 100 mgCOD/L was given. For each feast-famine cycle, the length of the feast and famine period was approximately 20 and 60 minutes, respectively. After the third famine phase, pulse wise feed-on-demand PHA accumulation was performed over 48 hours with constant mixing and aeration (Pei et al., 2022b). Substrate doses in pulse wise inputs targeted a maximum reactor acetic acid concentration of 100 mgCOD/L.

At selected time points 4 x 50 mL representative mixed liquor grab samples were taken. Duplicate sub-samples of 5 mL were fixed with formaldehyde to a final concentration of 3.7% (Sigma-Aldrich, Netherlands), preserved in 10 mL 1X phosphate buffered saline

(PanReac AppliChem, ITW Reagents, Spain) with 10 mL pure ethanol (VWR, the Netherlands), and stored at 5 °C before performing the staining. Afterwards, the remaining fixed sample was stored at −20 °C for long-term preservation.

4.2.2. Analytical Methods

Mixed liquor sample volumes were measured (V). Mixed liquor suspended solids were separated by centrifugation (3248 RCF and 4 °C for 20 min), and the supernatant was removed. The pellet mass was transferred with Milli-Q water (Merck, Germany) by rinsing into a clean pre-dried (105 °C) and tare weighed crucible. The pellet mass (m_1) representing total suspended solids (TSS) was estimated from the dried weight (105 °C over 12 hours). Afterwards, pellet fixed solids (m_2) were determined by ashing at 550 °C for 2 h. The mixed liquor VSS concentration was estimated as $(m_1 - m_2)/V$.

Biomass PHA content was determined by thermogravimetric analysis (TGA) according to Chan et al. (2017) with minor modification. The mixed liquor sample was acidified to nominally pH 2 with 98% H_2SO_4 (VWR, the Netherlands), mixed(10 min), and then centrifuged (3248 RCF and 4 °C for 20 min). The pellet was dried (105 °C overnight) and milled to a powder after decanting supernatent. About 5 mg of powder samples were disposed to thermal decomposition (TGA 2, Meller Toledor, Switherlands) in 3 principal steps as follows: 1) isothermal nitrogen gas atmosphere drying at 105 °C for 10 min; 2) heating with nitrogen gas atmosphere at 10 °C/min to 550 °C; 3) isothermal ashing in air atmosphere at 550 °C for 30 min.

The biomass PHA content as gPHA/gVSS was derived from the background corrected characteristic polymer decomposition peak detectable from the derivative thermogravimetric trend as described in Chan et al. (2017) using in-house Matlab data processing algorithms (MathWorks, MA, USA). Non-PHA biomass was estimated as VSS minus PHA mass. Subsequently, the mass ratio of PHA and non-PHA biomass was calculated.

4.2.3. Biomass Staining and Microscopy Image Analysis

For the samples fixed with formaldehyde, intracellular PHA granules and protein staining was performed using BODIPY 493/503® (BODIPY) (Thermo Fisher Scientific, MA, USA) in combination with SYPRO™ Red (Thermo Fisher Scientific, MA, USA) as described in Pei et al. (2022b). Glass slides with 10 reaction wells (Paul Marienfeld GmbH & Co.KG, Germany) were used. In each reaction well, 5 μL fixed sample was loaded with

0.5 μL BODIPY at 2 ng/μL and 0.5 μL of 100 times diluted SYPRO Red. The loaded slides were incubated at 46 °C until dried. The dried slide was washed with Milli-Q water to remove any excess dye and then dried again with compressed air. Each prepared slide was mounted with VECTASHIELD® HardSet™ Antifade Mounting Medium H-1400-10 (Vectashield) (Vector Laboratories, CA, USA) and sealed.

For the same series of the fixed samples, Fluorescence *In-situ* Hybridization (FISH) using the bacterial probe EUB338-I (5' GCT GCC TCC CGT AGG AGT 3') labelled with Cy5 was combined with PHA staining via BODIPY and DNA staining with DAPI. The staining was performed according to Llobet-Brossa et al. (1998) with modifications as described in Pei et al. (2022b). A 0.5 μL aliquot fixed sample was heat fixed in a slide well then dehydrated with ethanol. Hybridization buffer, 10 μL with 35% formamide, was added followed by 0.5 μL of EUB338-I, BODIPY and DAPI at, respectively, 50 ng/μL, 2 ng/μL and 250 ng/μL. The hybridization was performed at 46 °C for 1.5 h followed by washing using buffer pre-heated to 48 °C for 15 min and cold Milli-Q water. The washed slide was dried with compressed air, then mounted and sealed with Vectashield.

The Confocal Laser Scanning Microscope LSM 880 (Carl Zeiss, Germany) was used with Plan-Apochromat 10X/0.45 M27, 20X/0.8 M27 and 63X/1.4 Oil DIC objectives (Carl Zeiss, Germany). From each sample well, with 63X objective, a sequence of 11 randomly selected images with floc containing fields of view were acquired (digital resolution 1584 by 1584 pixel for the images with 2 dyes and 1912 pixel by 1912 pixel for the images with 3 dyes) after initially surveying the well for sample quality. Dyes were excited and dye specific captured images averaged from 16 scans were saved to separate file channels at 16 bit depth. To excite DAPI, BODIPY, SYPRO Red and Cy5, a Diode 405-30 laser at 405 nm, an Argon laser at 488 nm, a DPSS 561-10 laser at 561 nm and a HeNe633 laser at 633 nm were used, respectively. The same laser power and gain were used for each dye in each respective set of 11 images taken, and imaging conditions were otherwise kept similar from well-to-well.

Fiji Image J (ImageJ2, Ver 1.52P) was used for processing the captured images. Brightness levels were maximized without overexposing pixel data. Then for each dye specific channel with each respective well-series of 11 images, a cut off threshold intensity level was established by visual inspection. Total pixel areas of BODIPY, SYPRO Red, RNA FISH and DAPI were measured representing areas/bio-volume of PHA, proteins, 16S rRNA, and DNA.

4.2.4. Data Analysis and Interpretation

The trends in progress of biomass PHA content (gPHA/gVSS) over time were estimated by least-squares regression analysis according to Bengsston et al. (2017):

$$\text{Biomass PHA Content} = A_0 + A_1(1 - e^{-t/\tau}) \tag{4.1}$$

where A_0 and A_1 are constants that enable the estimation of rates as a function of time. The accumulation time constant τ (h) represented process first order kinetics in reaching a maximum level of PHA content.

From microscopy and image analysis, a characteristic relative area ratio for PHA to non-PHA biomass ratio (v/v) was calculated:

$$\text{PHA to non-PHA Biomass ratio (v/v)} = \frac{\text{PHA Area}}{\text{Non-PHA Biomass Area}} \tag{4.2}$$

where the overlay of PHA pixel area distribution on flocs were evaluated from the BODIPY signal. The non-PHA biomass area could be assessed from protein or DNA related fluorescent signals, namely Protein Area, and DNA Area.

Relative signal pixel distributions in overlay areas defined by the individual biomass flocs were used to give an index representing the fraction of the viable microorganisms as:

$$\text{Viable Microorganism Fraction} = \frac{\text{RNA Area}}{\text{DNA Area}} \tag{4.3}$$

Similarly, an index representing a measure for the PHA-storing microorganism fraction within the expressed viable areas for flocs within each captured field of view was characterized as:

$$\text{Viable PHA-storing Microorganism Fraction} = \frac{\text{PHA Area}}{\text{RNA Area}} \tag{4.4}$$

In order to impose a normal distribution for statistical analysis, the obtained v/v ratios were transformed to a logit scale (Warton et al., 2011):

$$x' = ln\frac{x}{1-x} \tag{4.5}$$

where x is the v/v ratios of PHA to non-PHA Biomass, Viable Microorganism Fraction and Viable PHA-storing Microorganism Fraction obtained by Equation (4.2), Equation (4.3) and Equation (4.4), and x' is the respective transformed v/v ratios.

The statistical analysis including one way ANOVA, Pearson's Chi-square normality test, quantile-quantile plot, linear regression were performed in the logit scale. The averages and the standard deviations for the ratios (Equation (4.2), Equation (4.3) and Equation (4.4)) were calculated with the logit transformed results (x'). Final results are reported with values transformed back to the scale of proportions (0-1):

$$X = \frac{10^{x'}}{10^{x'} + 1} \tag{4.6}$$

where x' is the average v/v ratios of PHA to non-PHA Biomass, Viable Microorganism Fraction and Viable PHA-storing Microorganism Fraction in the logit scale.

4.3. Results and Discussion

The potential for quantitative image analysis was evaluated from replicated accumulation experiments for the reproducibility and selection of image magnification. The PHA accumulation process performance was assessed by the biomass PHA content development (Equation (4.1)). From the optimal magnification, correlation between volume and mass ratios of PHA to non-PHA biomass was established. The degree of enrichment of PHA-storing bacteria for activated sludge from Bath WWTP was estimated. Subsequently, from the estimated biomass PHA content and the degree of enrichment, the average biomass PHA content for the PHA-storing fraction in the activated sludge was calculated. Combined staining of PHA, FISH and DNA was applied to monitor the trend of microbial activity during the PHA accumulation process.

4.3.1. Quantification of PHA Distribution

Previously methods of selective staining were developed and applied to visualise the PHA storage process (Pei et al., 2022b). Efforts to extend methods for quantitative image analysis started with the assessment of reproducibility alongside considerations for optimal image magnification. For the CSLM system, available objectives with 10, 20 and 63X magnification using optimum settings with 2 image channels, represented image

pixel dimensions of 0.26, 0.15 and 0.09 μm/pixel, respectively. Replicate slides (n=3) were prepared with 4 replicate wells per slide on biomass samples with negligible polymer content (0 h accumulation) and with significant polymer content (24 h accumulation). For each well, 4 to 11 fields of view were acquired at the three available magnifications Figure 4.1. Less images were acquired with 10X magnification compared to 63X where 11 images were taken due to floc concentration and differences in coverage. This dataset was used to evaluate quantitative reproducibility.

The pixel area of PHA is expected to be proportional to an integrated volume of PHA defined by the magnification dependent pixel area and CSLM focal depth. Similarly, the volume of non-PHA biomass is expected to be proportional to the protein area or DNA area identified with SYPRO Red or DAPI, respectively. These volumes relate to mass by respective densities of the biomass and PHA granules. If the densities remain constant during the accumulation process then the PHA to non-PHA biomass volume to volume ratio (v/v) from image analysis should correspond directly with the PHA to non-PHA biomass mass to mass ratio (m/m) provided by TGA analysis. However, the mass ratio can range from 0 to infinity while the volume ratio derived from overlapping respective pixel areas from image analysis is constrained to range from 0 to 1. This constrained range also affects the normality of the obtained results (Warton et al., 2011). To make the correlation between the volume ratio and the mass ratio, a logit transformation (Equation (4.5)) was applied to the data of image analysis to account for the inherently bounded scale.

After the logit transformation, the reproducibility of the volume ratio between the area of PHA and non-PHA biomass represented by the area of protein stain was assessed. For initial samples (0 hour) or samples after accumulation (24 hours), one way ANOVA of the averaged PHA and non-PHA biomass (protein) volume ratio per reaction well indicated no significant difference (95% CI) for analysis of replicate wells (n=4) on the same slide. Similarly, no significant difference (95% CI) was found among the reaction wells among three replicate slides. This outcome suggested that one well with a representative sample could describe the average state of the biomass during an accumulation if sufficient fields of view are acquired per well. Consistent outcomes between replicate slides supported potential for the quantitative reproducibility of the methods of staining and image analysis.

Influences of magnification on the observation of PHA-rich biomass were first assessed qualitatively. Details of interest including individual bacterial cells and PHA granules were anticipated to be in the order of 1 μm and 0.5 μm, respectively. As shown in Figure 4.1, the applied magnification for analysis did not strongly influence outcomes of image

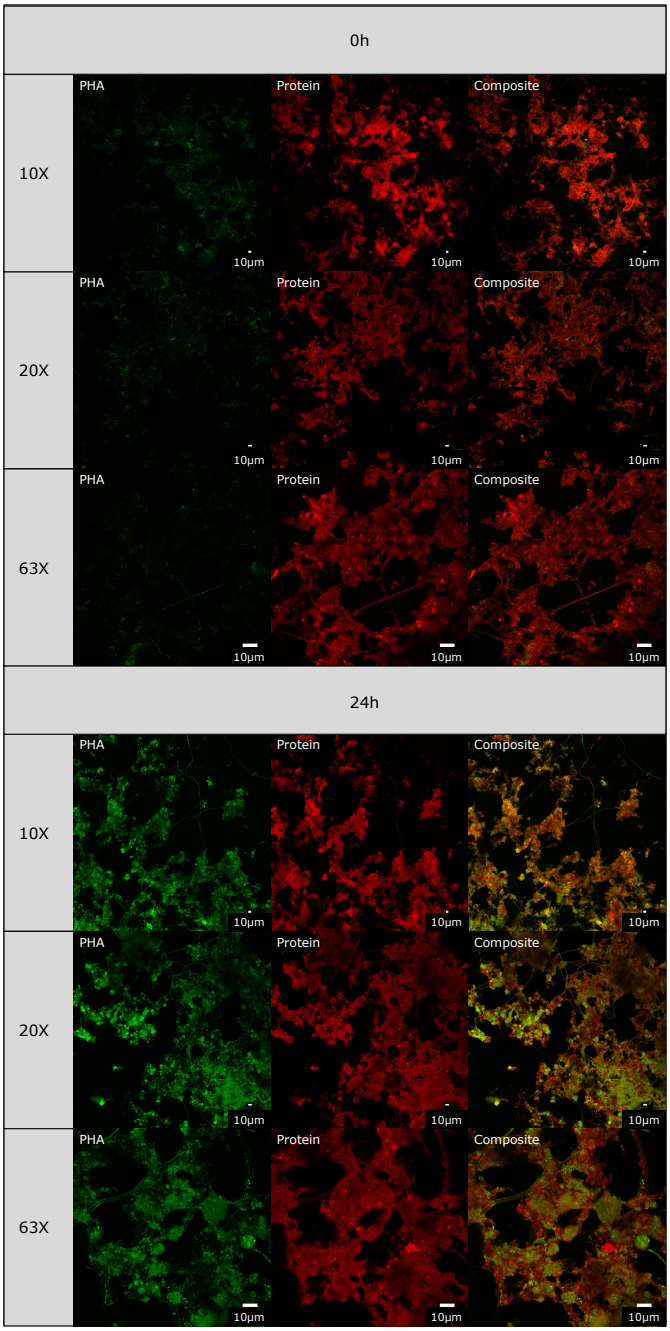


Figure 4.1: Activated sludge after 0 h and 24 h accumulation staining image channels for PHA (green) and protein (red), plus the composite image at 10X, 20X and 63X magnifications.

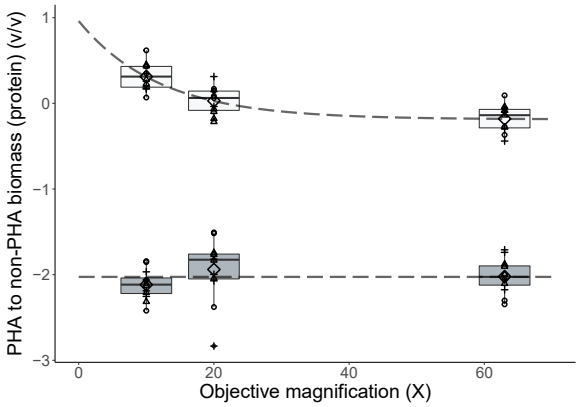
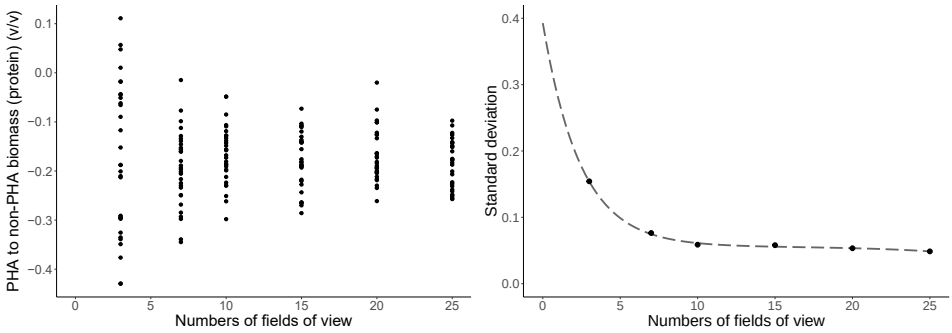


Figure 4.2: Box plot of volume ratio (Equation (4.2)) of PHA to non-PHA biomass (protein) for activated sludge in the logit scale after 0 h (gray) and 24 h (white) accumulation with pooled results from replicate slides (n=3) with 4 measurement wells on each slide, and 11 fields of view for each well with 10X, 20X and 63X magnification. The average volume ratio of PHA to non-PHA biomass (protein) for each slide 1, 2, 3 and the ensemble average are represented by ◯, △, + and ◇, respectively.

analysis at the beginning of an accumulation, because biomass PHA content is low at start. Even with 10X magnification, the morphology of microorganisms and the floc structures were clearly visible. After 24 h, with a pronounced degree of PHA accumulation, resolution of PHA with respect to biomass pixel areas was dependent on the magnification (Figure 4.1). A lower magnification provides a greater observation area with greater depth of field but PHA granules and individual cells were not well-resolved. With increasing magnification from 0.26 μm/pixel to 0.09 μm/pixel resolution, boundaries between cells and even between the intracellular PHA granules became increasingly more discernible. For example, considering filamentous microorganisms (Figure 4.1 (e)) the area occupied by PHA is smaller than biomass area defined by protein staining. It was observed that with lower magnification, interpreted extracellular polymeric substances surrounding flocs diffuse the signal by scattering signal from PHA. This reduced image quality and resulted in harder to determine area boundaries.

The potential for an influence from magnification on the quantitative image analysis was considered (Figure 4.2). At low biomass PHA content, even if average values may suggest a drift of underestimation, one way ANOVA results on the logit scale suggested no significant difference of the means (95% CI). For the PHA-rich biomass after 24 h accumulation, the average volume ratio of PHA to non-PHA biomass (protein) on the logit scale suggests a trend of a significant overestimation at lower magnification (DF=2, Residue= 26, F=27.48 ,

p<.001). Such bias may be due to pixel areas over representing the underlying PHA granule area with the decreased resolution. The normality of the PHA to non-PHA biomass volume ratio of 24 h samples obtained with different magnifications was tested using Pearson's Chi-square test. The ratios obtained when using 63X objective (p=.67) showed a better normality compared to 10X (p=.07) and 20X (p=.02) objectives were used. Therefore, considering the resolution and the outcomes for a normal distribution of the obtained results, 63X magnification was selected in subsequent development for a quantitative analysis.



(a) **(b)**

Figure 4.3: The Monte Carlo simulated average volume ratio of PHA to non-PHA biomass (protein) **(a)** and standard deviation of the simulated volume ratio of PHA to non-PHA biomass (protein) **(b)** using random selection (3, 7, 10, 15, 20 and 25 fields of view) of the pooled replicate data for 63X magnification and accumulated activated sludge after 24 hours.

A disadvantage of using higher magnification for the image acquisition and quantitative analysis was that each field of view captures a smaller absolute area. Due to an observed heterogeneity in floc to floc PHA granule distribution, sufficient fields of view were required towards obtaining a representative average value (Pei et al., 2022b). Monte Carlo simulation was performed for random selection of the pooled replicate data for 63X magnification and 24-hour accumulation (Figure 4.3). Results of Monte Carlo simulation, Figure 4.3 (a) suggested an increasing number of fields of view estimated PHA to non-PHA biomass ratios distributed in smaller ranges. Figure 4.3 (b) shows the standard deviation of the simulated results given in Figure 4.3 (a) when using different numbers of fields of view. The standard deviation indicated that increasing the acquired number of fields of view resulted in progressive reduction in the uncertainty of an estimated volume ratio of PHA to non-PHA biomass (protein). The trend suggests that for 63X magnification, at least 10 fields of view were required for reaching a low and steady standard deviation. When the numbers of fields of views increased from 3 to 10, the standard deviation decreased

from 0.15 to 0.06. When the numbers of fields of view further increased from 10 to 20, the standard deviation decreased from 0.06 to 0.05. Considering the trade off between the workload, the benefits, and the necessary redundancy, 11 fields of view were implemented in the following sections for a robust estimate of the average v/v distribution in the biomass.

4.3.2. PHA Accumulation Processes Performance

To assess if the staining method could follow the development of the PHA accumulation process quantitatively, 4 replicate 48-hour PHA accumulation experiments were conducted and evaluated. As shown in Figure 4.4, the trends of TGA measured biomass PHA content exhibited the robust performance with minor differences in accumulation extent even for these different batches of full-scale waste activated sludge collected on different days over 5 weeks. The trend in biomass PHA content development in time could be described according to Equation (4.1) with A_0, A_1 and τ equal to 0.02 gPHA/gVSS, 0.48 gPHA/gVSS and 9.1 h with the standard errors of 0.009, 0.010 and 0.720, respectively (on average with pooled data). From this development it was estimated that the biomass PHA content became essentially constant by 27 h (3τ). An average estimated biomass PHA content in replicate accumulations after 27 h was 0.49±0.03 gPHA/gVSS. The average maximum biomass PHA content was similar to what has been previously reported for this particular activated sludge (Bengsston et al., 2017). The average observed yield on substrate from the 4 accumulation experiments was also similar and equal to 0.27±0.03 gPHA/gAcetate (COD basis).

4.3.3. Quantitative Image Analysis

Samples were taken during all accumulation experiments, fixed, and stained according to developed methods (Pei et al., 2022b). The images were at 63X magnification assessed by CSLM with 11 random fields of view per well. The average of these 11 images was taken to represent average PHA to non-PHA biomass volume ratio. Floc to floc PHA distribution was found to be heterogeneous and variable. The correlation between the PHA to non-PHA biomass volume ratio and mass ratio was assessed by Pearson correlation test. The Pearson correlation test showed that the ratio of PHA to non-PHA biomass volume represented by protein area (Cor=0.90, DF=46, $p<0.001$) and DNA area (Cor=0.81, DF=45, $p<0.001$) gave correspondence with PHA to non-PHA biomass mass ratio (Figure 4.5 and Figure 4.6).

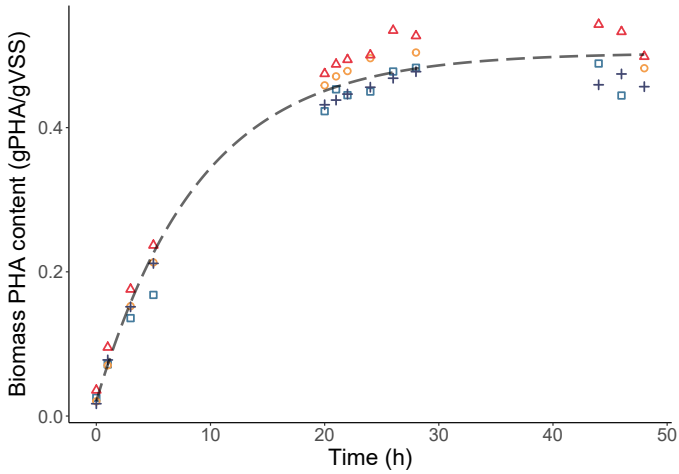


Figure 4.4: TGA measured biomass PHA content at different time point in 4 PHA accumulation replicated runs. Blue □, yellow ◯, red △ and purple + represent Run1, Run2, Run3 and Run4, respectively. The dash line ($y = 0.02 + 0.48 \times (1 - e^{-t/9.1})$) is the PHA content trend on average from 4 experiments with Equation (4.1).

The slope of the correlations is expected to be related to both measurement related factors as well as a density ratio conversion factor from v/v to w/w measurements. Different fluorophores have different characteristic imaging properties such as quantum yields. The outcome is that the more intense a signal the higher the probability that the fluorescence signal will diffuse and illuminate other surrounding Z stack layers of the floc structures. By CLSM, thin slices of the specimen are analyzed, with 1 Array unit setting for the CLSM, the integrated slice thickness depends directly also on the applied laser wavelength. Thus, as estimated in the appendix, the depth resolution decrease is in order of DAPI (258 nm), BODIPY (296 nm), SYPRO Red (355 nm) and Cy5 (388 nm). Measurement related factors may impose a systematic bias on the correlation, and they can also contribute to amplification of the observed variability depending on floc to floc differences in compactness.

Another factor that can affect the slope of the correlations is density differences between the PHA and non-PHA biomass. These differences generate a correction factor between the expressed mass ratio by TGA and the bio-volume ratios. The density of dried PHB powder is expected to be 1.25 g/cm^3 and non-PHA biomass had a density of around 1.01 g/cm^3 (Andreadakis, 1993; Peeters et al., 1994). However, the amorphous PHA and the nature of the PHA-rich biomass structures when hydrated is expected to influence density significantly. Neglecting the above mentioned laser dependent effects, volume

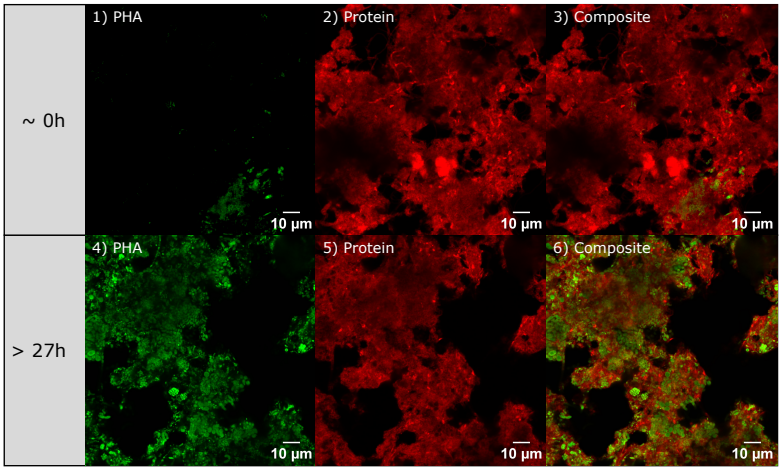
ratio of PHA to non-PHA biomass (protein) correlation coefficient suggests that the PHA granules would be about half the density of the non-PHA biomass (Figure 4.5). The suggested density difference would infer that for the downstream processing, the PHA-rich fraction of the biomass could be separated selectively by for example gradient centrifugation. More importantly, it suggested that the PHA could be harvested from the lighter fraction which agreed with Oshiki et al. (2010) where more PHA was recovered from the light fraction using buoyant density separation.

DNA staining and protein staining showed significant but also different correlation constants in the v/v to w/w relationship. The PHA to non-PHA biomass ratio was considered to be more closely reflected by PHA area and protein area because cell protein is more abundant and is distributed more widely. Interestingly, development of intracellular PHA granules could be observed to take space in the cell cytoplasm and displace DNA (Pei et al., 2022b). This effect was considered to be important especially when observing thicker floc structures. In these cases, PHA and DNA features were not well captured within the same focal plane affecting the representation the respective stained areas. However, overall, both the DNA and protein staining methods were capable of following the relative importance of v/v PHA accumulation in the biomass.

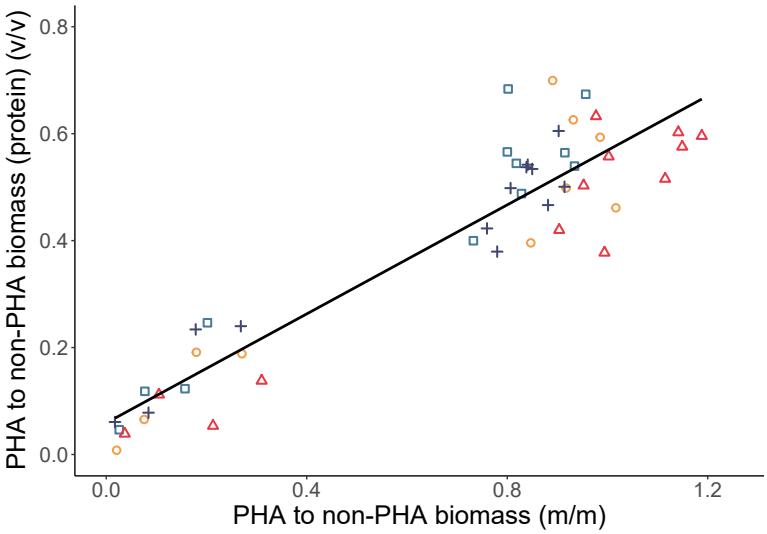
4.3.4. Degree of Enrichment for PHA-storing Microorganism

Both PHA to non-PHA biomass mass and volume ratios increased over time during the PHA accumulation process. At the later stage of the accumulation, stained PHA almost occupied the whole cell area to almost completely overlap with the biomass signal (Pei et al., 2022b). Therefore, the volume ratio of PHA to non-PHA biomass at later stage was used to approximate the degree of enrichment for the PHA-storing biomass fraction.

Since the mass of PHA approached its extant maximum after 27 h accumulation (Figure 4.4), results of v/v were pooled for an improved estimate of the degree of enrichment for PHA-storing microorganisms in the activated sludge. The degree of enrichment for PHA-storing microorganisms was estimated based on the applied staining pair, BODIPY with SYPRO Red or BODIPY with DAPI. The degree of enrichment estimated based on protein and DNA after 27 h, was found to be described by a normal distribution on the logit scale (Figure 4.7). The average degree of enrichment (logit scale statistics) estimated based on protein for waste activated sludge from Bath WWTP was 55%. The range from the back transformed logit based standard deviation was between 47% to 62% v/v.

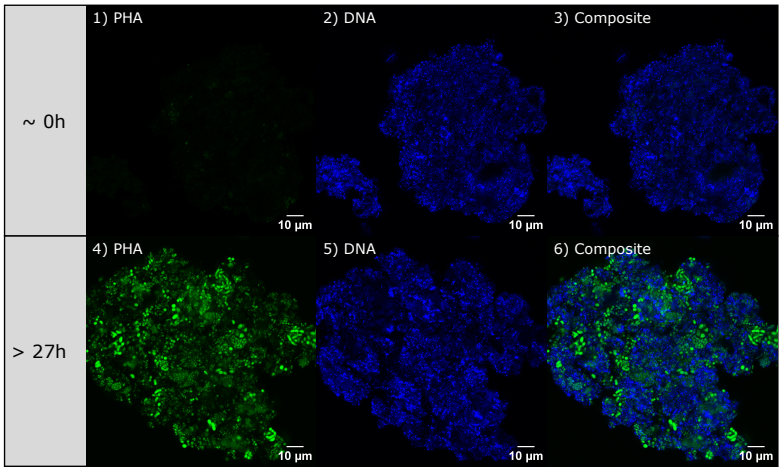


(a)

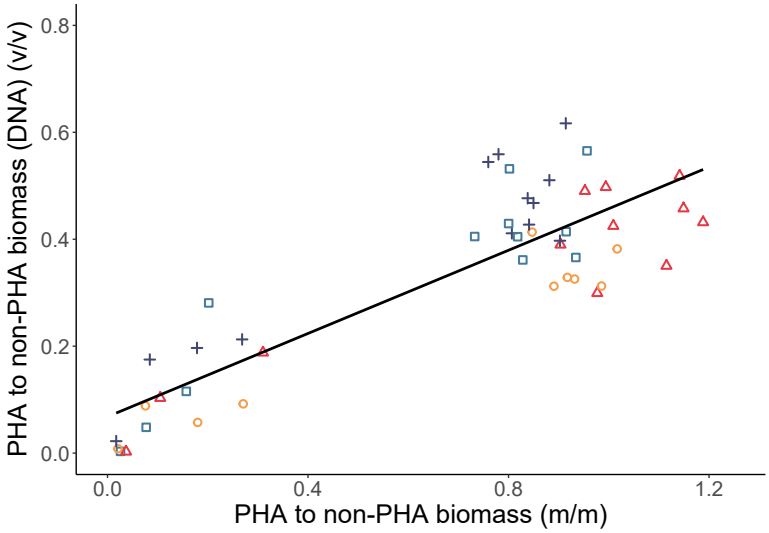


(b)

Figure 4.5: Typical representations of staining image channels for PHA (green) and protein (red), plus the composite image at 63X magnifications **(a)** for activated sludge with low PHA to non-PHA biomass ratio (0 h) and high PHA to non-PHA biomass ratio (>27 h) accumulation. The average PHA to non-PHA biomass mass ratio and the volume ratio of PHA to non-PHA biomass (protein) measured by staining and Confocal Laser Scanning Microscope (CLSM) in 4 PHA accumulation replicated runs **(b)**. Blue □, yellow ◯, red △ and purple + represent Run1, Run2, Run3 and Run4, respectively. The black line is the linear regression ($y = 0.51x + 0.06$, $R^2 = 0.86$) between PHA to non-PHA biomass mass ratio and the volume ratio of PHA to non-PHA biomass (protein).



(a)



(b)

Figure 4.6: Typical representations of staining image channels for PHA (green) and DNA (blue), plus the composite image at 63X magnifications **(a)** for activated sludge with low PHA to non-PHA biomass ratio (0 h) and high PHA to non-PHA biomass ratio (>27 h) accumulation. The average PHA to biomass mass m/m ratio versus the v/v ratio expressed by PHA area and DNA area **(b)**. Blue □, yellow ◯, red △ and purple + represent Run1, Run2, Run3 and Run4, respectively. The black line is the linear regression ($y = 0.39x + 0.07$, $R^2 = 0.74$) between between PHA to non-PHA biomass mass ratio and the volume ratio of PHA to non-PHA biomass (DNA).

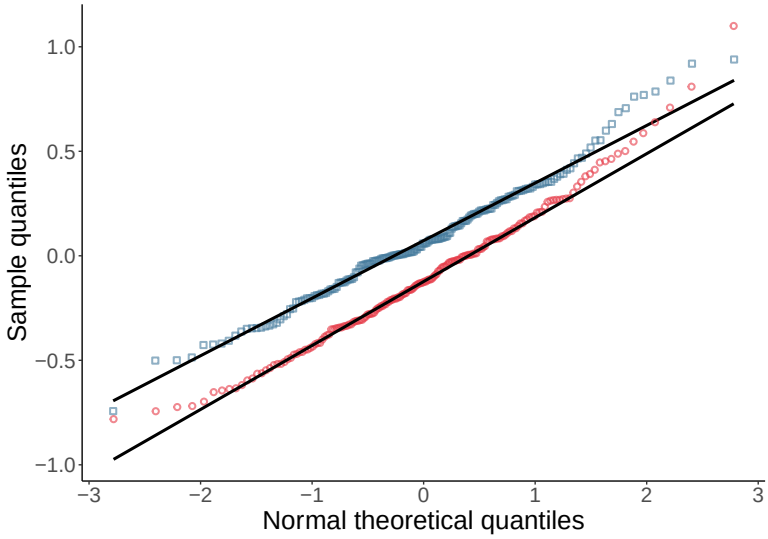


Figure 4.7: Quantile-quantile plot for the pooled data of degree of enrichment identified with protein (blue □) and degree of enrichment identified with DNA (red ◯)levels after 27 h on the logit scale.

The interpreted degree of enrichment for PHA-storing microorganisms of waste activated sludge from Bath WWTP indicates that only 55% of the biomass was accumulating PHA. This level is a significantly lower degree of enrichment compared to results reported for *enrichment accumulation* where the degree of enrichment was indicated up to almost 100% (Crognale et al., 2019). Therefore, waste activated sludge would perform better with *direct accumulation* if more of the organisms would accumulate PHA.

It has been suggested that possibilities to produce a better functional biomass in municipal activated sludge readily exist by methods to improve the stringency of disposing the biomass to periodic feast environments during the WWTP process (Morgan-Sagastume et al., 2014). Improvements in selection pressure without need for major process upgrades are expected to bring significant improvements in degree of enrichment without impacting on the core contaminants removal function of WWTP. For example, it has been shown at pilot scale how more stringent anoxic feast (pre-denitrification for a BNR process) increased biomass PHA content from 0.15 gPHA/gVSS to 0.49 gPHA/gVSS (Bengsston et al., 2017). However, in this past work a distinction was not made between feast pressures to induce microbial community changes for PHA-storing microorganism enrichment versus performance of the PHA-storing microorganisms in the biomass. The methods of the present investigation enable to evaluate enrichment distinct from accumulation

potential.

The observed heterogeneous nature of distribution of PHA in the sludge flocs is also informative towards development of efficient process strategies to recover the polymers. This work found that 45% of the biomass was void of polymer. Research efforts have been applied previously to upgrade a PHA-rich biomass based on density differences. Selective protein digestion, or mild non-specific oxidative treatment of the floc structures may also be expected to be effective pre-treatment if the non-PHA biomass fractions are more selectively influenced. At the same time, stress imposed on the biomass is known to promote polymer degradation. This risk for losses must be considered at the same time (Obruca et al., 2018). The advantage of selective staining methods is that such pre-treated biomass may also be evaluated towards a qualitative or quantitative impression of the morphological influence as a function of pre-treatment severity.

4.3.5. The PHA-storing Fraction

The estimated average PHA content in PHA-accumulating fraction was defined as follows:

$$\frac{\text{Biomass PHA content}}{\text{Biomass PHA content} + \text{DE} \cdot \text{Non-PHA biomass}} \tag{4.7}$$

where Biomass PHA content after 3τ is used and DE is the degree of enrichment defined by Equation (4.2). In the present study, average biomass PHA content for the PHA-storing fraction in the activated sludge was equal to 0.64 ± 0.04 gPHA/gVSS.

The estimated average PHA accumulation capacity for the PHA-storing fraction in these *direct accumulation* experiments was higher than the PHA accumulation capacity found for *enrichment accumulation* reported by Valentino et al. (2019a). However, the average PHA accumulation capacity for the PHA-storing fraction found in these *direct accumulations* was still lower than the more functionalized *enrichment accumulation* methods that select for *Plasticicumulans acidivorans* (Johnson et al., 2009). Therefore, the PHA accumulation capacity for the selected PHA-storing bacteria also influences the PHA accumulation potential.

These differences of the PHA accumulation capacity in both *enrichment* and *direct* accumulation suggested that selection of PHA-storing microorganisms does not necessarily

mean the selection of the accumulators with high PHA accumulation potential. Therefore, the function of the selection needs to be further understood and clarified without ambiguity with respect to effects of both degree of enrichment and PHA accumulation capacity for the PHA-storing fraction in the biomass.

Selection and resultant phenotype potential is one aspect of expressing a high biomass PHA content in a microbial community. Physiological state has also been reported to be of influence to the expressed biomass PHA content for given microorganisms or microbial communities (Morgan-Sagastume et al., 2017; Obruca et al., 2017). A given feast-famine regime or feed on demand strategy may be applied which induces optimum response for PHA accumulation. Often there are small differences in operational conditions that are not considered explicitly in reported comparisons of results. These differences may include biomass history in the WWTP before the accumulation process. Specific details also include the substrate supply during the accumulation process, background substrate levels during the accumulation process, the sizes or concentration of the pulses, and acclimation. Insights from evaluations based only on the end-point biomass PHA content are limited without reference to degree of enrichment. Therefore, it is important to incorporate the methods like selective staining to elucidate deepened understanding of operational conditions on the maximum biomass PHA content.

4.3.6. Quantification of the Dynamics of Microbial Activity Expressed by 16S rRNA

Dynamics of microbial activity were assessed by FISH targeting at 16S rRNA incorporated with PHA staining and non-PHA biomass counter staining. The area ratio between RNA and DNA was used to represent the viable bacteria fraction (Equation (4.3)). In the *direct accumulation* process, the viable bacteria fraction identified from individual fields of view at 63X magnification showed significant differences during the accumulation process (DF=12, Residue=568, F=12.04, p<.001). This significant difference suggested that within the biomass, the microbial activity is distributed heterogeneously among flocs.

The average activity for the biomass was calculated by making an average of the RNA to DNA ratio for 11 fields of view from one well with 63X magnification. This average activity for the biomass did not follow a measurable trend during the whole accumulation process (DF=12, Residue=34, F=1.503, p=0.171). The interpreted average microbial activity levels for the biomass were stable, without correlation to time within the precision of these

evaluations. A specific activity level influence of acclimation before accumulation was also not found. To increase the resolution of the methods, it is necessary to identify PHA accumulation activity level at the cellular level. This level of identification would require techniques such as applying qPCR to the expressed PhaC gene, FISH targeting at mRNA of PhaC, or BONCAT-FISH targeting at expressed PHA polymerise proteins.

The staining tools in combination with FISH could be applied to follow the dynamic nature of the biomass and discriminate between active PHA-accumulating, active non PHA-accumulating and non-active organic fractions of the activated sludge. In the waste activated sludge collected from Bath WWTP, based on the replicate experiments of distinct batches, the activated sludge was fractionated. The average viable biomass fraction during the whole accumulation process (evaluated on the logit scale) was 70% with a range from the back transformed logit standard deviation of 52% to 83% RNA/DNA. The area ratio between PHA and RNA was used to represent the PHA-accumulating fraction of the viable biomass (Equation (4.4)). It was found that 68% (PHA/RNA) of the active biomass was accumulating PHA. Combining the size of active biomass fraction and its PHA-accumulating fraction, it was estimated that 48% of non-PHA biomass (based on DNA staining) was actively accumulating PHA. Therefore, as shown in Figure 4.8, the non-PHA biomass for activated sludge from Bath WWTP could be fractionated into 48% of active PHA-accumulating fraction, 22% of active non PHA-accumulating fraction and 30% non-active organic fraction. The active non PHA-storing fraction dilutes not only the biomass PHA content but also consumes substrates and oxygen. This active flanking population could contribute to lowering PHA yield, and overall process performance/productivity.

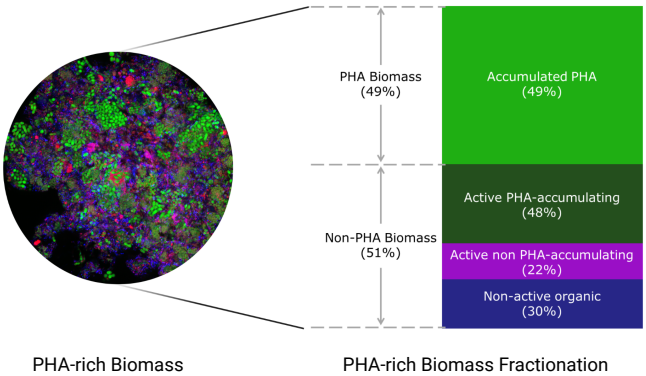


Figure 4.8: The PHA-rich biomass fractionation based on the staining of PHA (green), RNA (red) and DNA (blue). The figure was created with *BioRender.com*.

4.4. Conclusions

- Quantitative image analysis methods for evaluating PHA storing microbial communities have been developed. They allow for the estimation of the PHA storing bacteria fraction and the average PHA content of the PHA storing bacteria.
- The waste activated sludge used in these *direct accumulation* experiments expressed a degree of enrichment of 55% (47%-62%).
- The PHA in the activated sludge was diluted by flanking biomass. The estimated average biomass PHA content for the PHA-storing biomass fraction was 0.64 ± 0.04 gPHA/gVSS.
- *Enrichment accumulation* approach has higher degree of enrichment but is not necessarily enriching bacteria that express a higher PHA accumulation capacity compared to *direct accumulation*.
- Selection has two components: to improve the degree of enrichment and to promote conditions to select PHA-storing bacteria with a higher PHA accumulation capacity.
- Microbial activities in the total biomass were relatively constant during the accumulation process and 68% of active bacteria were estimated to be accumulating PHA. This outcome confirmed the existence of viable flanking microbial population which may consume added substrate and generate additional oxygen demand in a PHA accumulation process.

Appendix

According to the manual of Zeiss for LSM 880, while using 1 Array Unit, the optical slice thickness and axial resolution are identical. This is often referred as depth resolution and can be calculated with the following equation with a correction factor of 0.87:

$$\frac{0.64 \times \overline{\lambda}}{n - \sqrt{n^2 - NA^2}}$$

Where, $\overline{\lambda} = \sqrt{2}\frac{\lambda_{Ex}+\lambda_{Em}}{\sqrt{\lambda_{Ex}^2+\lambda_{Em}^2}}$, could be simplified as $\overline{\lambda} = \sqrt{\lambda_{Ex} \times \lambda_{Em}}$;

n: refraction index;

NA: numerical aperture.

The depth resolution was estimated for the dyes while using objective 63X/1.4 Oil DIC M27 that $NA = 1.4$. In this case, the applied immersion oil Immersol 518 F (Carl Zeiss, Germany) had a refraction index $n = 1.518$. For the estimation of the $\overline{\lambda}$, the wavelength of the laser for exciting the respective dye was used as λ_{Ex} and the maximum emission reported by the manufacture was used as λ_{Em}. The estimated $\overline{\lambda}$ and depth resolution are summarized in the following table:

Table 4.1: The estimated depth resolution for DAPI, BODIPY, SYPRO Red and Cy5 using objective 63X/1.4 Oil DIC M27 in combination with immersion oil Immersol 518 F.

	Ex (nm)	Em (nm)	$\overline{\lambda}$ (nm)	Depth resolution (nm)
DAPI	405	461	432	258
BODIPY	488	503	495	296
SYPRO Red	561	630	594	355
Cy5	633	666	649	388

Acknowledgement

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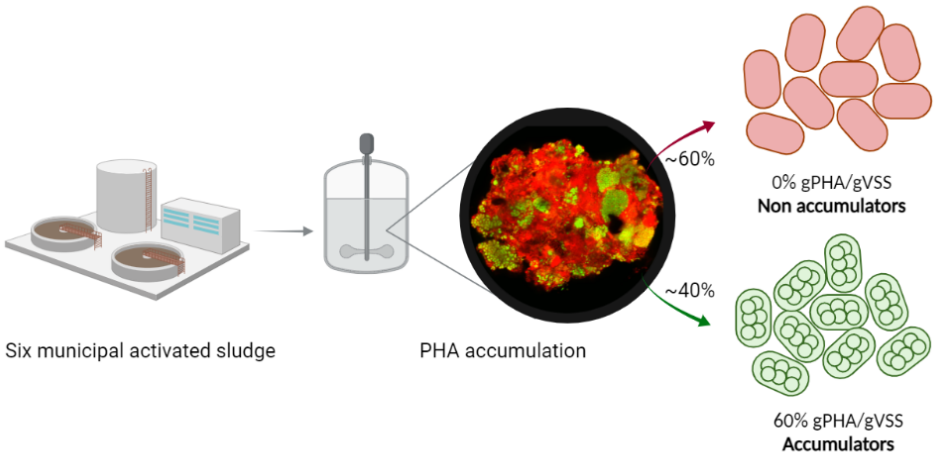
5

Exploring the Limits of Polyhydroxyalkanoate Production by Municipal Activated Sludge



[1]This chapter has been published: Pei, R., Estévez-Alonso, A., Ortiz-Seco, L., Van Loosdrecht, M. C. M., Kleerebezem, R., & Werker, A. (2022a). Exploring the Limits of Polyhydroxyalkanoate Production by Municipal Activated Sludge. *Environmental Science & Technology*. https://doi.org/10.1021/ACS.EST.2C03043

Highlights

- *PHA contents between 0.18 and 0.42 gPHA/gVSS were obtained with activated sludge*
- *The degree of enrichment for PHA-storing bacteria ranged from 0.16-0.51 (v/v)*
- *PHA-storing bacteria in activated sludge accumulated up to 0.58 ± 0.07 gPHA/gVSS*
- *Degree of enrichment is the key factor to reach PHA contents up to 0.6 gPHA/gVSS*
- *Reaching 0.6 gPHA/gVSS can expand direct use of activated sludge for PHA production*

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.

Keywords: *resource recovery, municipal wastewater treatment, activated sludge, biopolymers, polyhydroxyalkanoate (PHA).*

5.1. 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 (Kehrein et al., 2020; Wagner et al., 2002). Different process configurations have evolved over the last century to efficiently remove these contaminants, involving nitrification-denitrification and enhanced biological phosphorus removal (Lofrano et al., 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 (Cydzik-Kwiatkowska et al., 2016; Lu et al., 2014; McIlroy et al., 2015). 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 (Majone et al., 1999; Van Loosdrecht et al., 1997). 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 (Mino et al., 1998; Seviour et al., 2003; Van Loosdrecht et al., 1997). 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 (Á. Estévez-Alonso et al., 2021a; Kourmentza et al., 2017). 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 (Bengsston et al., 2017; Beun et al., 2000). 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 (Á. Estévez-Alonso et al., 2021a; Kourmentza et al., 2017). 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 (Bengsston et al., 2017).

Determination of the biomass (average) PHA content is most commonly reported on a mass basis (Kourmentza et al., 2017; Reis et al., 2003), 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., 2022b; Pei et al., 2022c). 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 intrinsic 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.

5.2. Materials and Methods

5.2.1. Sludge Source and Feedstock

Grab samples of activated sludge from six different municipal WWTP were used for standardised PHA accumulation assays (Table 5.1). 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 (Bengsston et al., 2017). 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.

Table 5.1: 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

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.

5.2.2. 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., 2017). 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 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_La). 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).

5.2.3. 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 μL were loaded in reaction wells (10 per glass slide). Reaction wells were further provided with 0.5 μL BODIPY at 2 ng/μL and 0.5 μL 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:

$$\text{PHA to non-PHA biomass ratio (v/v)} = \frac{\text{PHA Area}}{\text{Protein Area}} \tag{5.1}$$

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.

5.2.4. 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 μm 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 (Á. Estévez-Alonso et al., 2021b).

One of the 15 mL aliquots was used for PHA determination. The liquid volume 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).

5.2.5. Data Analysis

All measured parameters were corrected for effects of sample withdrawal and feedstock addition from liquid and mass balance considerations (Johnson et al., 2009). 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 Bengsston et al. (2017):

$$\text{Biomass PHA content} = A_0 + A_1 \left(1 - e^{-k_1 t}\right) \tag{5.2}$$

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 τ ($\tau = 1/k_1$ (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 0.2τ and 3τ, respectively. The times of 0.2τ and 3τ were when biomass reached 18% and 95% of maximum PHA content, respectively. In assays where 3τ 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/gX_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:

$$\text{PHA to non-PHA biomass ratio (v/v)} = B_0 + B_1\left(1 - e^{-k_2 t}\right) \tag{5.3}$$

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:

$$\text{Average PHA content in PHA-accumulating fraction} = \frac{\text{Biomass PHA content}}{\text{Biomass PHA content} + \text{DE} \cdot X_a} \tag{5.4}$$

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.

5.3. Results

The outcomes for the standardised PHA accumulation assays from the six sources of municipal activated sludge are summarized in Table 5.2. From these assays, the degree of enrichment for PHA-accumulating biomass fraction for the activated sludge was determined results are given in Table 5.3.

Table 5.2: 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. 0.2τ and 3τ were when biomass reached 20% and 95% of maximum PHA content, respectively.

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

5.3.1. 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 5.1). 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 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 slowly (Equation (5.2)). COD mass balances that were estimated from measured and estimated values could not be closed. Initially (0.2τ) and at the later stages (3τ 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.

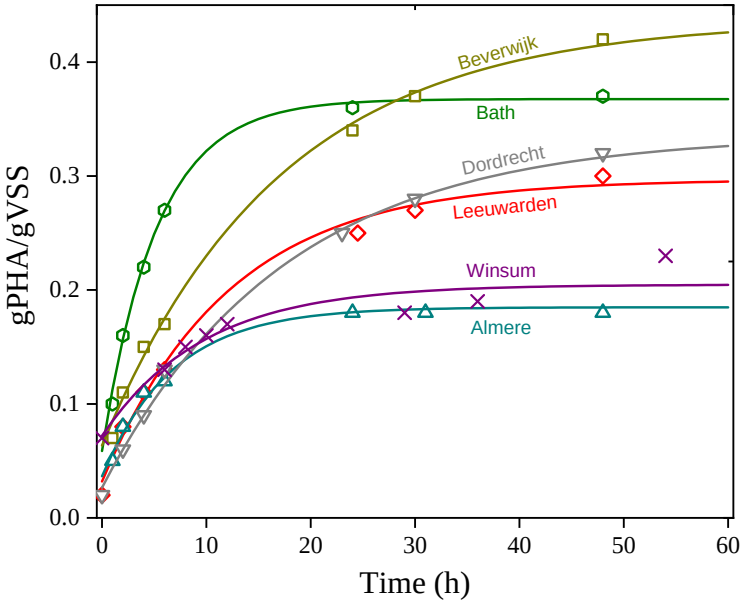


Figure 5.1: PHA accumulation trends for all the assayed WWTPs. Symbols represent the measured values and the trend lines are from Equation (5.2).

5.3.2. 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 5.2. 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.

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.

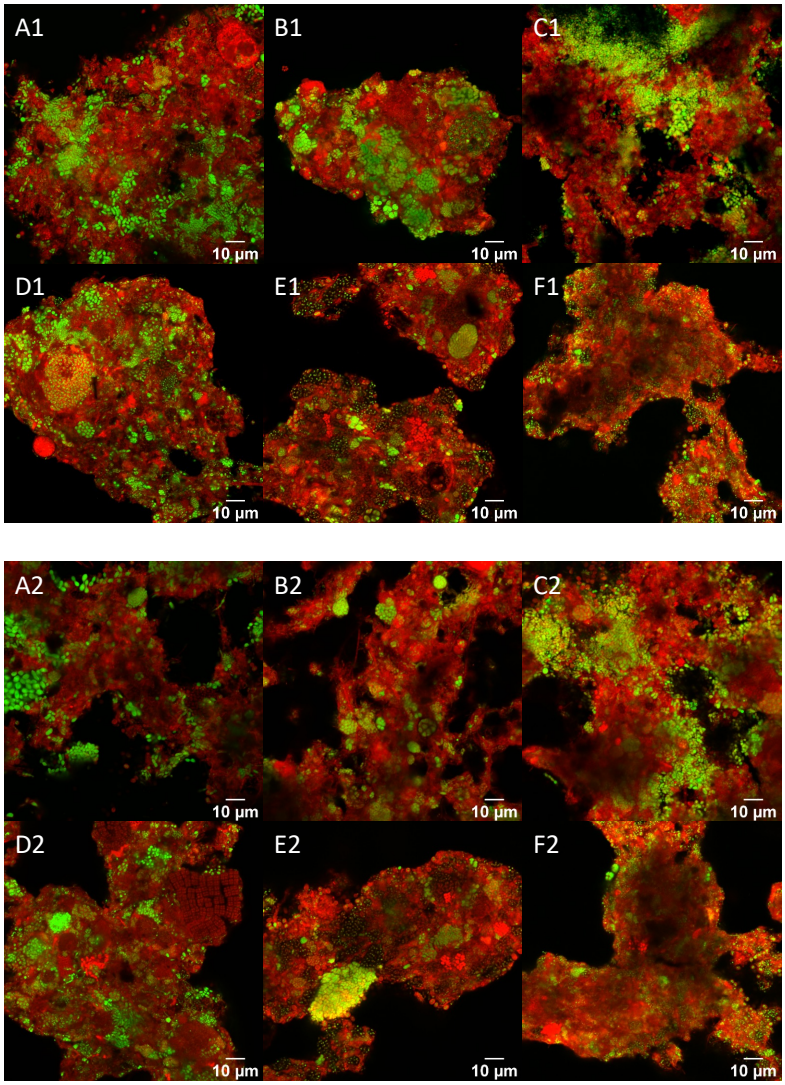


Figure 5.2: Stained PHA granules (green) and non-PHA biomass (red) after 48 h accumulation from WWTP of Bath (A), Leeuwarden (B), Bevewijk (C), Almere (D), Dordrecht (E) and Winsum (F) at different fields of view.

5.3.3. Degree of Enrichment and Average PHA Contents in the PHA-accumulating Fraction

Figure 5.2 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 (5.3), as observed in Figure 5.3. 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 content and degree of enrichment from six municipal activated sludge sources replicated the experience previously observed by Pei et al. (2022b), Pei et al. (2022c) 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

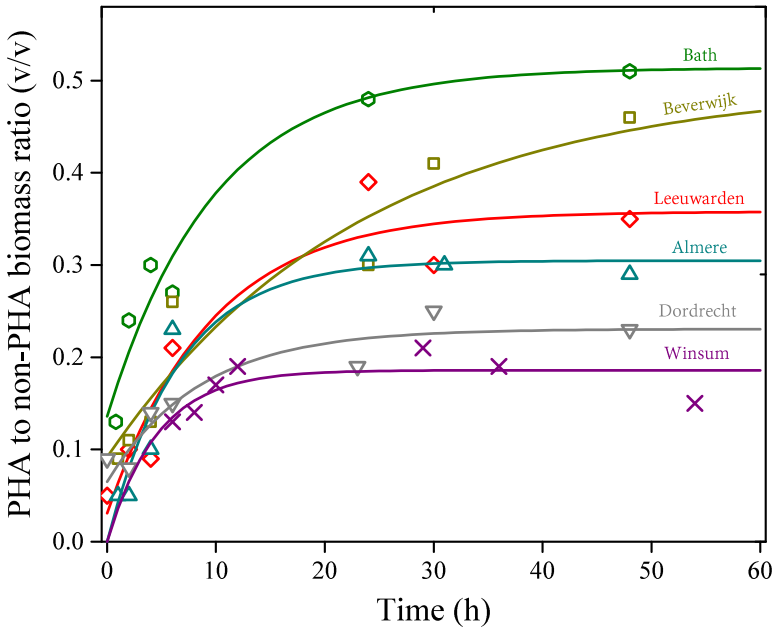


Figure 5.3: Development of PHA to non-PHA biomass ratio (v/v) during PHA accumulation assays. Symbols are the measured values from image analyses and the trend lines are from Equation (5.3).

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 (5.4)). 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.

5.4. Discussion

5.4.1. 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 (Á. Estévez-Alonso et al., 2021a; Kourmentza et al., 2017; Valentino et al., 2017). 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 (Arcos-Hernández1 et al., 2015; Bengsston et al., 2017). 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., 2009). 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 (Á. 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

Table 5.3: 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

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 (Arcos-Hernández1 et al., 2015; Cavaillé et al., 2013; Chinwetkitvanich et al., 2004; Liu et al., 2011). If the upper limits (0.6 to 0.7 gPHA/gVSS) can be consistently 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 (Crognale et al., 2019; Á. Estévez-Alonso et al., 2021a; Frison et al., 2015; Kourmentza et al., 2017; Moretto et al., 2020b). 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., 2021; Stouten et al., 2019).

5.4.2. 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., 2017). 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 et al., 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., 2017). 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 (Bengsston et al., 2017). 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) (Bengsston et al., 2017). 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 (Jiang et al., 2011; Krishna et al., 1999; Stouten et al., 2019). 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).

5.4.3. 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 (Equation (5.2)). 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 (Cavaillé et al., 2013; Mulders et al., 2020b; Valentino et al., 2015). 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 increased 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.

Acknowledgments

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6

Influence of Process Conditions on Accumulated Polyhydroxybutyrate in Municipal Activated Sludge

A modified version of this chapter has been submitted.

Highlights:

- *Intracellular PHA depolymerase activity can be constrained anaerobically.*
- *Changes in environmental conditions cause polymer loss even for short term storage.*
- *Extracellular depolymerase activity is limited if the PHA remains intracellular.*
- *Acidic conditions are preferred to preserve quantity and quality for PHB recovery.*
- *The melting enthalpy of PHB in the biomass can indicate PHB extractability.*

Abstract *Polyhydroxybutyrate (PHB) was accumulated in full-scale municipal waste activated sludge at pilot scale. After accumulation, the fate of the PHB-rich biomass was evaluated over two weeks as a function of initial pH (5.5, 7.0 and 10), and incubation temperature (25, 37 and* 55 °C*), with or without aeration. PHA became consumed under aerobic conditions as expected with specific rates in the range of 0.16 to 0.39* d^{-1}*. Under anaerobic conditions, up to 63 percent of the PHB became consumed within the first day (initial pH 7,* 55 °C*). Subsequently, with continued anaerobic conditions, the polymer content remained stable in the biomass. Degradation rates were lower for acidic anaerobic incubation conditions at a lower temperature (*25 °C*). Polymer thermal properties were measured in the dried PHB-rich biomass and for the polymer recovered by solvent extraction using dimethyl carbonate. PHB quality changes in dried biomass, indicated by differences in polymer melt enthalpy, correlated to differences in the extent of PHB extractability. Differences in the expressed PHB-in-biomass melt enthalpy that correlated to the polymer extractability suggested that yields of polymer recovery by extraction can be influenced by the state or quality of the polymer generated during downstream processing. Different post-accumulation process biomass management environments were found to influence the polymer quality and can also influence the extraction of non-polymer biomass. An acidic post-accumulation environment resulted in higher melt enthalpies in the biomass and, consequently, higher extraction efficiencies. Overall, acidic environmental conditions were found to be favourable for preserving both quantity and quality after PHB accumulation in activated sludge.*

Keywords: *polyhydroxyalkanoate (PHA), polyhydroxybutyrate (PHB), waste activated sludge, biopolymer, downstream processing, polymer properties.*

6.1. Introduction

Polyhydroxyalkanoates (PHAs) are a family of naturally occurring polyesters that accumulate intracellularly in many species of microorganisms as an endogenous organic substrate (Dawes et al., 1973). These microorganisms accumulate PHA when they are subject to dynamic environments when, for example, surplus organic carbon sources become suddenly available while other growth factors, such as nitrogen and phosphorus, are limiting (Majone et al., 1999; Reis et al., 2003; Van Loosdrecht et al., 1997). PHAs can be accumulated in microbial biomass within engineered bioprocesses to significant levels (0.40 to 0.90 grams PHA per gram organic mass) (Á. Estévez-Alonso et al., 2021a). Accumulated intracellular PHA can be extracted and purified. Purified PHAs exhibit thermoplastic properties similar to fossil derived polyesters (Raza et al., 2018). Compared to fossil-based plastics, plastics made from PHA are bio-based and can be completely biodegradable. Thus, PHAs are an anticipated renewable resource for use in applications, especially where biodegradation is required.

PHAs are produced commercially today with pure culture methods, using refined substrates and sterilization. PHA plastics compete with a higher selling price compared to conventional petroleum derived plastics, and this limits PHA market penetration (Plastics Europe, 2020; Vandi et al., 2018). Cost reduction has been an underlying motivation driving much fundamental research and development in methods and processes for PHA production. Over the past two decades, much advancement has been made in the use of open culture production methods and waste organic residuals for substrates as a principal strategy to reduce PHA production costs (Á. Estévez-Alonso et al., 2021a; Reis et al., 2003).

Municipal biological wastewater treatment processes produce significant amounts of waste activated sludge. Municipal activated sludge can exhibit a significant degree of enrichment for the PHA-storing phenotype and can be directly used for PHA accumulation. (Á. Estévez-Alonso et al., 2021a; Pei et al., 2022c). PHA-accumulating bacteria can exist in the waste activated sludge due to the dynamics in the process environments or dynamics in daily loading conditions, which make metabolism involving polymer storage inherently a good selective strategy for survival (Van Loosdrecht et al., 1997). In recent years, technical viability for the use of waste activated sludge for PHA production has progressed from lab to pilot scale (Bengtsson et al., 2017; Á. Estévez-Alonso et al., 2021a; Valentino et al., 2018). Pilot scale production trials have exhibited stable outcomes in quantity and quality of polymers produced with average biomass PHA content with respect to volatile solids (VS) of 0.4 gPHA/gVS (Á. Estévez-Alonso et al., 2021a; Reis et al.,

2003). More recent investigations suggest expectations for optimal waste activated sludge accumulation to reach about 0.64 gPHA/gVS which is similar to typical expectations in general for both pure and open culture production methods alike (Pei et al., 2022a).

The direct use of activated sludge for PHA production has two main process components: PHA accumulation and downstream processing (DSP). PHA accumulation is an aerobic bioprocess where the activated sludge is fed with a volatile fatty acid (VFA) rich substrate to reach the maximum possible biomass PHA content. DSP starts after PHA accumulation with steps to separate process water and recover a purified PHA from the biomass suspended solids. DSP methods are less well understood for the up-scaling of open culture PHA production and are a current knowledge gap needed for industrial-scale developments (Á. Estévez-Alonso et al., 2021a). DSP steps are critical to preserving polymer quantity and quality within the harvested post-accumulation PHA-rich biomass and contribute heavily to the overall process economics (Kourmentza et al., 2017; Werker et al., 2020).

Recovery and purification method developments of PHA are ultimately water or solvent based (Burniol-Figols et al., 2020; Á. Estévez-Alonso et al., 2021a; Kosseva et al., 2018; Lorini et al., 2020; Ong et al., 2018; Werker et al., 2020). Water based methods work to recover the PHA granules from solubilized biomass solids (Kourmentza et al., 2017). Solvent based methods recover solubilized polymer from biomass suspended solids (Kourmentza et al., 2017). Published research developments for DSP are commonly focused on the specific purification steps. Towards this aim, comparisons are made for different approaches or conditions for smaller subsamples from a batch of PHA-rich biomass preserved directly after accumulation by laboratory scale centrifugation, followed by thermal drying or lyophilisation. However, the fate of the PHA directly after accumulation and the stability of the freshly made product have not been systematically studied.

Microorganisms mobilise PHA not only during starvation but also to mitigate stresses (Müller-Santos et al., 2021). Changes in environmental conditions (i.e. osmotic pressure, oxygen level, pH and temperature) after PHA accumulation can also promote PHA mass loss and property changes. Abiotic random scission due to β elimination in the presence of H^+ or OH^- will reduce molecular weight (Lauzier et al., 1994). Intracellular and extracellular PHA depolymerase enzymes are anticipated to still be active directly after the PHA accumulation process. Intracellular PHA depolymerase enzymes that are integral to the surface of the PHA granules (Jendrossek, 2009). They are reported to act on intracellular PHA in its so-called native amorphous hydrated state (Jendrossek, 2009; Oeding et al.,

1973; Ong et al., 2017). Meanwhile, extracellular depolymerase enzymes are understood to degrade PHA from denatured granules from lysed cells, and for this semi-crystalline polymer in nature (Jendrossek et al., 2002; Ong et al., 2017). Directly after the PHA accumulation process, in absence of any cell lysis, PHA will be stored intracellularly in host cells and act as bioactive functioning granules. Therefore, factors and environments that can influence the level of intracellular PHA depolymerase activity should, at least initially, determine the polymer fate.

The metabolism of intracellular PHA degradation is still not completely clear. For polyhydroxybutyrate (PHB), the depolymerase enzyme hydrolysis leads to hydroxybutyrate or hydroxybutyryl coenzyme A and then enters the β-oxidation cycle to be further metabolised (Eggers et al., 2013; Gebauer et al., 2006; Jendrossek et al., 2002; Uchino et al., 2007). The degradation process may require the activation of released hydroxybutyrate to hydroxybutyryl coenzyme A which is an energy-consuming reaction (Eggers et al., 2013). The intracellular depolymerase activity level can be influenced by the environmental conditions and/or the polymer properties. Environmental conditions such as temperature, pH, and oxygen concentration can affect enzyme production, activity, and stability. Due to enzyme substrate specificity, research on intracellular depolymerase properties requires native PHA granules, which can be challenging to isolate (Jendrossek et al., 2002). Hence, it is not straightforward to systematically evaluate optimum temperatures or pH levels for all possible and potentially different intracellular depolymerase activities.

During the initial steps of DSP, material management methods can include a need in the logistics to stage the freshly produced PHA-rich biomass with temporary storage. Handling and time scales of the industrial processes before the final DSP steps of purification can result in PHA mass and property losses. For example, even heating rates in biomass thermal drying significantly influence product molecular weight (A. Estévez-Alonso et al., 2022). The risk for polymer loss and properties changes directly after the PHA accumulation may be expected to be negligible in gram laboratory- and even kilogram pilot scale PHA production research and development work. Due to the relatively low volumes and masses of material to be handled, the steps of DSP can be managed quickly (minutes to a few hours). At the industrial scale, with significantly higher expected process volumes and masses of material to handle, duty cycles for each fresh batch may need longer (hours to days). PHA-rich biomass can be produced at a number of distributed sites, and at least some DSP steps, including final drying, can be handled more centrally with improved scales of the economy as done today for municipal sludge management. Therefore, the transport of dewatered PHA-rich biomass may benefit the production economics. Quicker

handling requires higher throughput (larger) equipment and associated capital costs. Smaller equipment would require that PHA-rich biomass be temporarily stored in DSP feed tanks.

The stability of the PHA in the fresh crude product of accumulated PHA-rich biomass has not been reported in the research literature. The effects of time and environmental conditions in the storage of PHA-rich biomass fate are overlooked. Therefore, it was of interest to explicitly evaluate how well freshly accumulated PHA survived this stage with environmental conditions from acidic to basic pH, mesophilic to thermophilic temperatures, and with or without the presence of oxygen. For the present investigation, the effect of environmental conditions (pH, temperature, aeration) on the fate of freshly accumulated PHA in municipal activated sludge was evaluated. A pilot scale process was operated with an acetic acid feedstock to yield a PHB-rich biomass. The harvested PHB-rich biomass mixed liquor was divided into parallel batches. Parallel batches were pH adjusted and incubated at selected temperatures, with and without aeration. Sampling and monitoring of polymer fate over time for mass balances and property evaluations were undertaken and interpreted as reported herein.

6.2. Materials and Methods

6.2.1. PHA Accumulation

Waste activated sludge was obtained from Bath Wastewater Treatment Plant (WWTP, Rilland-Bath, the Netherlands). This WWTP treats a mixture of municipal and industrial influent wastewater for 470,000 person equivalents. The WWTP includes primary treatment followed by a modified Ludzack-Ettinger activated sludge biological process. Phosphorus is removed by $FeCl_3$ precipitation. Batches of fresh grab samples of gravity belt thickened WAS (56.7 gTS/L) were taken and delivered on September 5th, November 4th and December 3rd, 2019. The thickened waste activated sludge was stored at 5 °C pending its use in accumulation experiments. Accumulation production experiments were performed with respective batches on September 25th, November 5th and December 4th, 2019. The freshly accumulated PHA-rich biomass was the starting material used for the principal incubation experiments. Control incubation experiments were also performed with the same activated sludge but without any PHA accumulation.

The feedstock for PHA-rich biomass production was 20 gCOD/L acetic acid (VWR, the Netherlands) with added NH_4Cl and KH_2PO_4 (VWR, the Netherlands) for a COD:N:P (by

weight) of 100:1:0.05. The pH was adjusted to 5.0±0.5 with NaOH (VWR, the Netherlands). Thus, the PHA produced was polyhydroxybutyrate (Lemos et al., 2006).

The pilot scale accumulation process was carried out in a jacketed stainless steel 200 L reactor with 167 L working volume as described in Pei et al. (2022a). Accumulation experiments were initiated by loading the reactor with a set weight of the thickened waste activated sludge that was diluted with tap water to a starting suspended solids concentration of about 4 gVSS/L. The reactor was operated at 25 °C with constant mixing (230 rpm) and aeration (50 L/min). Before the accumulation process started, a period of steady aeration was applied for about 12 h hours to reach a steady level of endogenous respiration. This steady level was determined from respiration levels based on the dissolved oxygen trends.

The accumulation process was fed-batch with a constant process volume and constant aeration. Aeration maintained dissolved oxygen levels over 1 mgO_2/L. Reactor volume continuous circulation through a clarifier (16 L) maintained a constant overall process volume with retention of the suspended solids. The substrate was pumped in discrete pulses of a fixed volume to target peak pulse substrate concentrations of 100 mgCOD/L. Pulse input timing was from feedback control that was triggered by respiration level monitoring based on dissolved oxygen trend measurements (JUMO ecoLine O-DO, JUMO GmbH & Co. KG, Germany) as previously described by Werker et al. (2020). The process started with an acclimation step (Morgan-Sagastume et al., 2017) using three acclimation pulses as previously reported (Pei et al., 2022b). After the third acclimation cycle, the accumulation process was directly started. During accumulation, feed pulses were given intact with the substrate uptake rate based on dissolve oxygen trends and the tight series of input pulses were thereby given without excess substrate supply and build up in the reactor over 20 hours.

6.2.2. PHA-rich Biomass Incubation Experiments

Three replicate batches of PHA-rich biomass were produced and used directly after 20 hours accumulation for the purposes of the incubation experiments. Accumulation was terminated by switching off aeration, but mechanical mixing was sustained to maintain a well-mixed liquor. After accumulations pH was nominally 8, and since a final pulse of the substrate was given before aeration was terminated, the initial background acetic acid concentration was about 100 mgCOD/L.

For each incubation experiment, three 10 L grab samples of mixed liquor were taken from the accumulation reactor. The initial pH of the grab samples was adjusted to pH 5.5, 7 and 10 using 1M HCl or 1M NaOH, respectively. During the incubation experiment, pH was thereafter monitored but not further controlled.

Respective batches of pH adjusted mixed liquor were then distributed in replicate 170 mL aliquots to 21 x 250 mL stoppered serum bottles and 9 x 500 mL Erlenmeyer flasks with sponge stopper. Serum bottle headspace was exchanged with nitrogen gas with overpressure to establish and maintain anaerobic incubation conditions. A Discofix® one-way Stopcock (B. Braun, Germany) together with a 40 mm needle was connected to each of the anaerobic serum bottles. This connection had a radially and axially movable swivel lock for a safe and quick connection, and it was used for liquid/gas sampling. Erlenmeyer flasks with porous sponge stoppers were used for aerobic incubations. Serum bottles and Erlenmeyer flasks were incubated on an orbital shaker table at 120 rpm and at a constant selected temperature (25, 37 or 55 °C).

The incubation period was for up to 15 days, for which during weekdays, daily grab samples were taken in triplicate using 3 of the incubation vessels for each condition defined by initial pH, incubation temperature, and with or without aeration. The remaining unsampled serum bottles provided a mass of material for end-point assessment with the characterisation of the resulting suspended solids and polymer quality.

6.2.3. Control Incubation Experiments

Three 1.3 L jacketed batch reactors were operated isothermally at 37 °C in batch mode under selected constant pH (5.5, 7 and 10). pH was monitored and controlled by dosing inorganic acid/base. The reactors were flushed with N_2 gas to ensure anaerobic conditions. An aliquot of nominally 200 g PHA-free waste activated sludge also from Bath WWTP was incubated with 2.5 g commercial PHB (>98% purity, Biomer Germany) with the particle size of 273±13 μm. In addition, about 30 g of inoculum from a full-scale anaerobic digestor was used to ensure the presence of active acidogenic activity. Two grams 2-bromoethanesulfonate were used to inhibit the potential for methanogenesis activity. These control experiments were to evaluate the fate of exogenous PHB due to extracellular polymerase activity as a function of initial pH.

Control experiments were also carried out by incubating pure (>98 wt %) commercial PHB powder (Biomer, Germany) at a concentration of 1 g/L in triplicate 100 mL with closed serum bottles. The initial pH in the serum bottle was around 7. The serum bottles

were incubated at 120 rpm and 37 °C. Hydroxybutyrate and VFA concentrations were monitored over time.

Another control experiment was conducted with pure (>98 wt%) commercial PHB powder (Biomer, Germany) at a concentration of 1 g/L and lipase enzyme Lipozyme® CALB (Novozymes, Denmark) from *Candida Antartica* B at a concentration of 10 mL/L in triplicate 100 mL closed serum bottles. The serum bottles were incubated at 120 rpm and 37 °C. Hydroxybutyrate and VFA concentrations were monitored over time.

6.2.4. Analytical Methods

Figure 6.1 shows schematically the sample analysis workflow. The mixed liquor pH (SevenExcellence S400 Basic, Mettler Toledo, Switzerland) and total Chemical Oxygen Demand (tCOD) were monitored. tCOD samples were diluted up to 10 times with Milli-Q water (Merck, Germany) for cuvette COD test kits (Hach Lange, USA).

Headspace samples were taken from the experimental stoppered serum bottles by syringe. Biogas formation was detected from gas chromatography (Varian CP-4900 Micro-GC, Varian, UK). The gas chromatograph was equipped with Mol Sieve 5Å PLOT (MS5) (10m x 0.53 mm, 30 μm, fused silica, aluminosilicate phase) column and a thermal conductivity detector.

For soluble water quality analyses, mixed liquor samples were centrifuged (3248 RCF for 20 min at 4 °C, Beckman Coulter, CA, USA). The pellet was saved for suspended solids quality analyses, and the decanted supernatant was filtered (1 μm GF/0.45 μm PVDF Phenex™-GF/PVDF Syringe Filters, Phenomenex, USA).

The filtered liquid samples were assessed for soluble Chemical Oxygen Demand (sCOD), VFAs, hydroxybutyric acid, anion, and cation concentrations. sCOD was determined with the COD cuvette test kits depending on the concentration (Hach Lange, USA). VFAs and hydroxybutyric acid concentrations were measured by Dionex ultra-high-pressure liquid chromatography system with Phenomenex Rezex organic acid H^+ column (300× 7.8 mm) and a Dionex Ultimate 3000 RS UV detector (210 nm) with 2.5 mM sulfuric acid mobile phase at a flow rate of 0.5 mL/min and 80 °C. Anions were determined by ion chromatography (Metrohm 761 Compact IC, Metrohm, Switzerland) with a built-in conductivity detector. A pre-column (Metrohm Metrosep A Supp 4/5 Guard) and a column (Metrohm Metrosep A Supp 5, 150/4.0 mm) were used with 3.2 mM sodium carbonate and 1 mM sodium bicarbonate + 1% acetone solution mobile phase at 0.7 mL/mL at

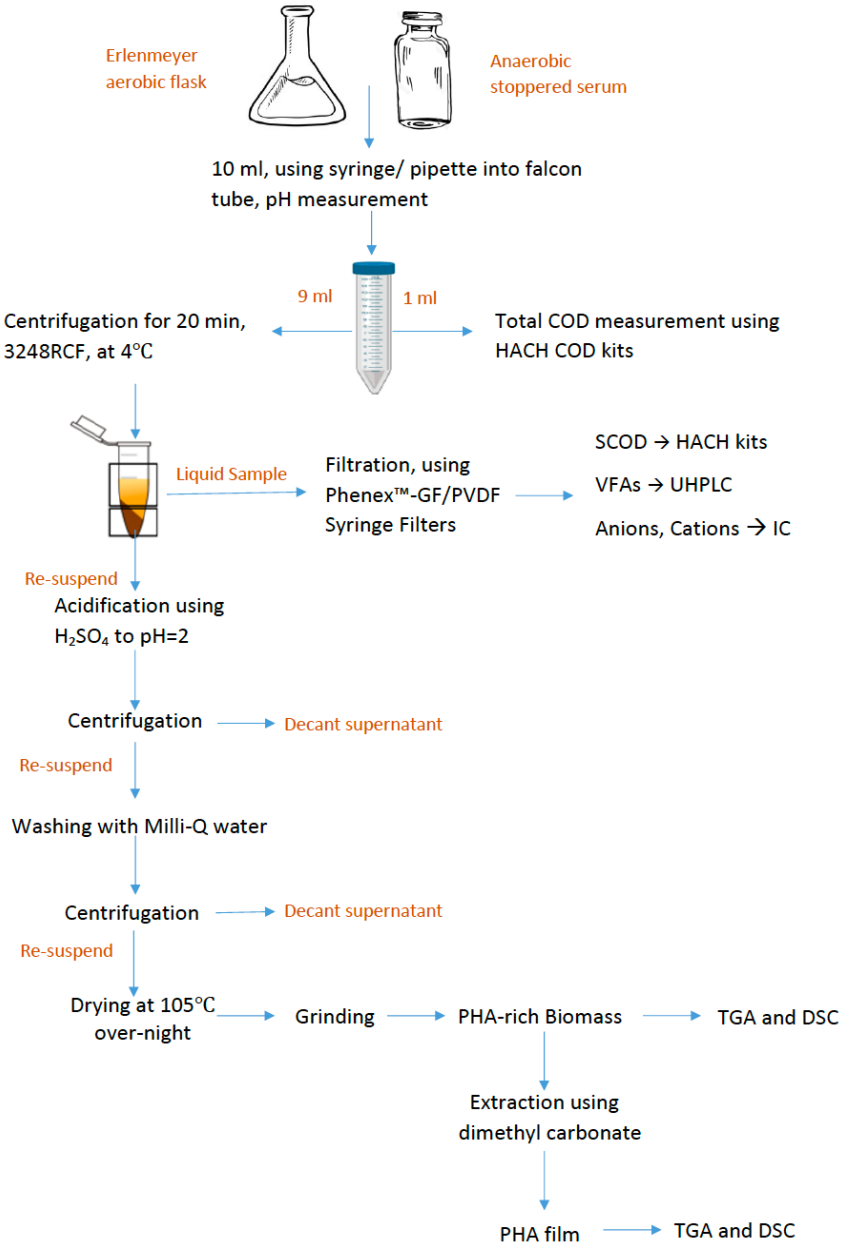


Figure 6.1: The sample preparation and analysis scheme.

room temperature. A chemical suppressor was also applied (0.2 M phosphoric acid + 1% acetone at 0.1 mL/mL). Ion chromatography was also used to assess cation concentrations (Metrohm Compact IC Flex 930 Metrohm, Switzerland). The ion chromatograph was equipped with a pre-column (Metrohm Metrosep RP 2 Guard/3.6), a column (Metrohm Metrosep C 4–150/4.0 mm) and a conductivity detector. HNO_3 with a concentration of 3 mM was used as a mobile phase at a flow rate of 0.9 mL/min.

The collected suspended solids pellet of PHA-rich biomass, after the centrifugation, was first re-suspended with Milli-Q water (Merck, Germany) and then acidified down to pH 2 by addition of 98% H_2SO_4 (VWR, the Netherlands). The acidified sample was mixed (5-10 min), centrifuged (3248 RCF for 5 min at 4 °C), and the supernatant was removed. The acidified pellet was again re-suspended with Milli-Q water to remove residual H_2SO_4, centrifuged as before, to re-collect the now acidified and washed pellet. The pellet was dried at 105 °C overnight (12 h), weighed, and then ground to a fine powder. These dried and ground biomass solids were characterized by TGA and DSC (see below).

Carbon, nitrogen, hydrogen, and oxygen composition for the PHA-rich biomass was measured by elemental analysis based on the modified Dumas Method (FlashSmart, Thermo Fisher Scientific, MA, United States). The elemental analyzer consisted of two furnaces, one for C/H/N/S measurements at 950 degrees and the other for oxygen measurement at 1060 degrees. A 140 mL/min carrier gas, a 250 mL/min oxygen gas, and a 100 mL/min reference gas were used.

PHA was extracted from the dried and ground solids in 13 mL dimethyl carbonate (DMC, Sigma-Aldrich ReagentPlus®, 99%) from ground biomass samples to target a maximum theoretical polymer concentration of nominally 20 mgPHA/mL but lower in some cases due to limitations in the available amount of sample. 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 a 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 RCF over 2 minutes) with pre-heated (90 °C) tube inserts to remove the 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 (Sartorius MA37). The 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 of biomass in the extraction tube after the initial re-drying before solvent addition. The polymer film thermal properties were characterised by TGA and DSC (as described below).

The dried ground biomass solids and the extracted PHA films were assessed by TGA (TGA 2, Metller Toledo, Switzerland) for biomass PHA content and thermal stability (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 the estimation of sample residual moisture/solvent and PHA contents as well as the overall dried sample organic and inorganic fractions. Briefly, pre-weighed samples were inserted into the TGA at 80 °C with nitrogen purge gas at 50 mL/min. The temperature was increased (10 °C/min) to 105 °C and held for 15 minutes drying, wherein removed moisture (or residual solvent) weight 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 from the weight lost after incubation at 550 °C with air atmosphere after 30 minutes. Reference samples included PHA-rich biomass with known PHA content (45.1 ± 0.6 gPHA/gVSS), and pure PHB (>98% purity, Biomer, Germany). Instrument temperature measurement was calibrated based on Curie temperature with a nickel standard following Mettler-Toledo methods. The biomass PHA content as gPHA/gVSS was derived from the background corrected characteristic polymer decomposition peak detectable from the derivative thermogravimetric trend as described in (Chan et al., 2017) using in-house Matlab data processing algorithms (MathWorks, MA, USA). Non-PHA biomass (NPB) was estimated as VSS minus PHA mass. Subsequently, the mass ratio of PHA and non-PHA biomass (f_{PHA}) was calculated.

Differential scanning calorimetry (DSC 3+, Mettler-Toledo, Switzerland) of the PHA in the dried biomass and extracted films was performed based on previously described methods (Chan et al., 2017). About 5 and 2 mg dried biomass and recovered polymer were used from each sample, respectively. Weighed samples in vented crucibles 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 crystallisation enthalpies were estimated with respect to the mass of PHA in the sample estimated from TGA measurements for the same samples. 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.

6.3. Results and Discussion

6.3.1. Incubation and Preservation of Accumulated PHB Quantity

Three batches of pilot scale PHA-rich biomass were produced. The measured maximum biomass PHA contents at the end of 20 hours of accumulation were 0.37, 0.53 and 0.50 gPHA/gVSS, respectively. Each batch of the PHA-rich biomass was characterized by elemental analysis to obtain a representative estimate of the NPB COD content for making COD mass balances. Acetic acid results in the accumulation of PHB, and the elemental composition of PHB is ($C_4H_6O_2$). For COD conversion of PHB, 1.67 gCOD/gPHB is applied. Combining information of VS, and PHB contents with elemental analysis, the non-PHA biomass compositions were estimated and found to be consistent between batches as shown in Table 6.1. The average COD of the NPB was found to be 1.36 gCOD/gNPB and this value was applied in the COD mass balance evaluations. The NPB composition and theoretical COD conversion factor are similar to what was reported in the literature ranging from 1.37 to 1.48 gCOD/gNPB (G.-H. Chen et al., 2020). This result suggested that the PHB accumulation process did not affect the overall active biomass composition.

Trends in development for biomass PHA content are typically expressed as PHA concentration over the total VSS concentration during accumulation in the research literature. This ratio is indicative of the performance of the active biomass during an accumulation process towards reaching a steady relative level of the accumulated polymer in the biomass. It is the value measured directly by TGA. Alternatively, the PHA to NPB ratio

Table 6.1: The VSS and PHB contents of the biomass TSS, and the estimated non-PHA biomass (NPB) elemental composition were used towards calculating the corresponding NPB COD conversion coefficient from three distinct batches of PHA-rich biomass.

	VSS/TSS %	PHB Content gPHB/gVSS	NPB Composition	Conversion Coefficient gCOD/gNPB
Batch 1	88.93	0.37	$CH_{1.7}N_{0.17}O_{0.55}$	1.35
Batch 2	90.76	0.53	$CH_{1.7}N_{0.18}O_{0.51}$	1.36
Batch 3	87.47	0.50	$CH_{1.7}N_{0.20}O_{0.48}$	1.37
Average				**1.36**

shows trends of the relative fate of PHA with respect to NPB. Biomass PHA content is converted to a PHA to NPB ratio as follows:

$$f_{PHA} = \frac{\text{PHA mass}}{\text{NPB mass}} = \frac{\text{PHA content}}{\text{1-PHA content}} \tag{6.1}$$

The PHA to NPB ratio on a COD basis was estimated with the conversion factors for PHB (1.67 gCOD/gPHB) and for NPB (1.36 gCOD/gNPB) from the elemental analyses.

The PHA to non-PHA biomass ratio (f_{PHA}) reported on the relative gain or loss of PHA with respect to non-PHA biomass solids during aerobic or anaerobic incubations. Typical results of trends are shown for the PHA to NPB ratios in the set of incubations for initial pH 7 for aerobic or anaerobic conditions at selected temperatures (Figure 6.2). Similar plots of results for the other initial pH sets of incubations are provided as supplementary information (SI).

Under aerobic conditions without further exogenous substrate supply after PHA accumulation, intracellular PHA is expected to be consumed. The trends of consumption, as shown in Figure 6.2 and SI were fitted to the model of a shrinking particle with least squares regression analysis. In this model, PHA removal rate decreases asymptotically following a 2/3 power law as a function of f_{PHA} (Tamis et al., 2014b). The integrated rate function solved for f_{PHA} is as follows:

$$f_{PHA} = (\sqrt[3]{f_0} - \frac{1}{3} k t \cdot \frac{C_{x0}^{\frac{1}{3}}}{C_x})^3 \tag{6.2}$$

where f_{PHA} is PHA to non-PHA biomass ratio (COD basis); f_0 is the initial PHA to non-PHA biomass ratio; $\frac{C_{x0}}{C_x}$ is the ratio between the initial and final non-PHA biomass. As illustrated

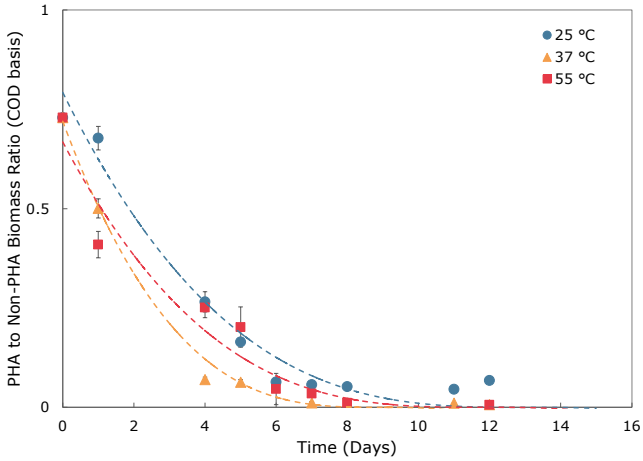
in Figure 6.2a and SI, and similarly for all the initial pH conditions and temperatures with aerobic incubation the shrinking particle model represented the decay of PHA mass to negligible levels. The aerobic groups served as the benchmark for the polymer fate due to an expected depolymerase activity stimulated by famine conditions and influenced due to different environmental conditions of initial pH and temperature (Tamis et al., 2014b).

The estimated rate constant k from the model distinguished between levels of depolymerase activity due to initial pH and temperature (Table 6.2). k ranged from 0.16 to 0.39 d^{-1} depending on the initial pH and temperature. For any given incubation temperature, alkaline pH consistently exhibited relatively higher k values. Initial mildly acidic or neutral pH suggested optimal depolymerase activity to be between 25 and 55 °C. Since rates at initial pH 10 were insensitive to temperature, the maximum aerobic depolymerase rate for this activated sludge is expected to be in the order of 0.38 d^{-1}. Mild acidic conditions and elevated temperatures also stimulated higher depolymerase activities relative to neutral initial pH conditions. Therefore for an activated sludge from a municipal wastewater treatment plant with operating pH between 7 and 8, and temperatures between 15 and 30 °C, mild stresses in pH and temperature, in combination with oxygen supply, are detrimental, to the objectives of polymer product conservation (Henze, 1997). However, if aeration cannot be avoided, then acidic conditions and lower temperatures are preferred.

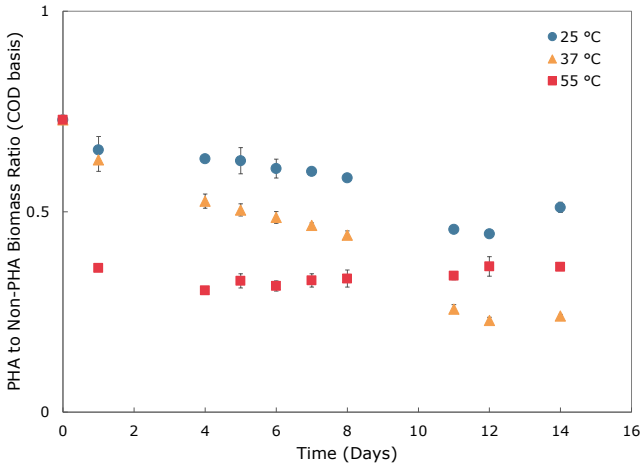
Table 6.2: The rate constant k of aerobic incubation at different initial pH and temperature derived by the shrinking particle model described by Equation (6.2) (Tamis et al., 2014b).

	Initial pH 5.5	Initial pH 7	Initial pH 10
25 °C	0.16	0.19	0.38
37 °C	0.32	0.27	0.38
55 °C	0.26	0.18	0.39

Using the same starting PHA-rich biomass, the incubations under strictly anaerobic conditions showed a different kind of characteristic trend (Figure 6.2b) compared to the aerobic benchmark cases (Figure 6.2a). Since the polymer metabolism due to depolymerase activity requires energy, anaerobic conditions under famine were expected to inhibit excessive polymer loss as predicted by the aerobic model described by Equation (6.2). However, on the contrary, a relatively sudden initial loss in the relative amount of PHA was observed over the first day before the PHA level with respect to non-PHA biomass became stabilised over at least 8 days of continued incubation. Anaerobic conditions did promote the preservation of the intracellularly stored polymer quantity but not as



(a)



(b)

Figure 6.2: The trends of PHA to non-PHA biomass ratio (f_{PHA}) over time for an initial pH of 7 with incubations at 25, 37 and 55 °C aerobically (a) and anaerobically (b). Error bars report the standard deviation between 3 replicate samples from respective incubation tubes. Symbols cover error bars in some cases.

strictly as was anticipated. In contrast, anaerobic conditions with exogenously supplied PHB in control experiments resulted in rapid complete removal of added polymer powder after an initial lag phase with an initial pH of 7 (SI). The lag phase was longer, and the degradation rate was slower, given an initial pH of 10. The lag phase was prolonged for this with an initial pH of 5.5. The exogenous polymer persevered better under acidic conditions than the endogenously stored polymer. However, neutral initial pH favoured more rapid exogenous polymer consumption.

Even under anaerobic conditions, a limited amount of the accumulated polymer became rapidly mobilized. To estimate for differences in the effect of environmental conditions on the initial relative anaerobic PHB loss, a degradation rate over the first day was estimated for f_{PHA} and is summarised in Table 6.3. The activated sludge used in the present study was a nitrification-denitrification biomass. This activated sludge is anticipated to be dominated by aerobic or facultative microorganisms. Therefore, anaerobic conditions would be expected to inhibit depolymerase activity due to a lack of energy flow to kick off the metabolism (Jendrossek, 2009; Oeding et al., 1973; Ong et al., 2017). Lower temperatures and neutral or mild acidic conditions offered the greatest degree of preservation of polymer. One of the possible reasons for higher or lower initial albeit limited degradation rate under anaerobic conditions could be due to an imposed environmental stress on the freshly accumulated PHA-rich biomass. To deal with stress, microorganisms may mobilize PHA (Müller-Santos et al., 2021). This ability and extent for apparent mobilization seem to be further influenced by initial pH and temperature. Under alkaline conditions, rates of loss are indicated to be highest between 25 and 55 °C. However, under neutral or mildly acidic conditions, the degree of mobilization is observed to have increased with temperatures up to 55 °C, and decreased with lower pH. These results support that lower pH is preferred to limit the extent of polymer mobilization. However, the extrapolated trend does suggest that an initial rate is not eliminated at pH 2 even if acidification to pH 2 has become a commonly applied practice prior to final dewatering and drying (Bengsston et al., 2017).

Table 6.3: The PHB degradation rate (%/day) over the first day under anaerobic conditions.

	Initial pH 5.5	Initial pH 7	Initial pH 10
25 °C	-0.13	-0.17	-0.50
37 °C	-0.25	-0.17	-0.70
55 °C	-0.32	-0.63	-0.42

The PHA to non-PHA biomass f_{PHA} indicated the depolymerase activity level and the

relative change of PHA. If anaerobic incubation were effective in solubilizing NPB, then f_{PHA} would be expected to increase in time. However, in the balance after initial PHB losses and up to 8 days of incubation, the amount of PHA relative to NPB remained stable.

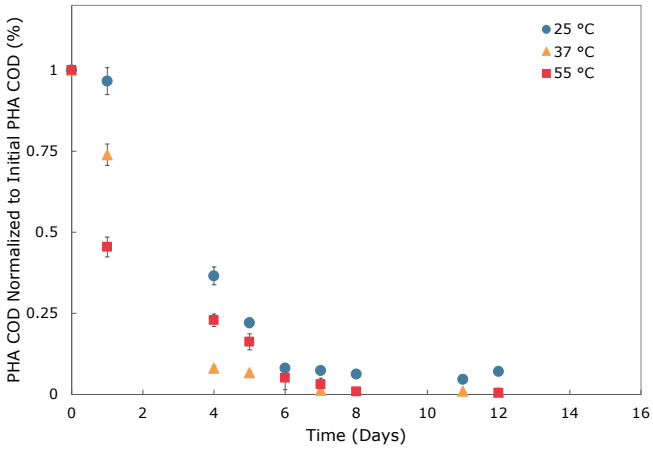
A COD mass balance on PHB was applied to determine if this stability corresponded to the absolute or relative preservation of the accumulated polymer in the PHA-rich biomass. The concentration of polymer for each sampling time was calculated from the overall mass balance:

$$\mathrm{tCOD} = \mathrm{sCOD} + \mathrm{COD}_{\mathrm{NPB}} + \mathrm{COD}_{\mathrm{PHB}} \tag{6.3}$$

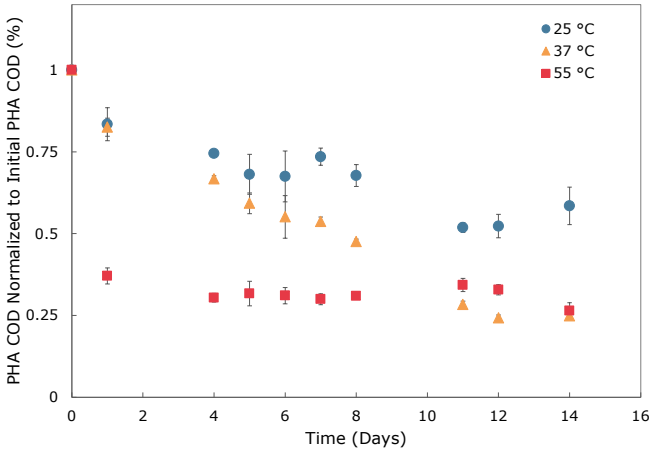
where tCOD and sCOD were measured, and the COD ratio of PHA and NPB were determined from TGA measurements coupled to PHB and estimated NPB COD content per unit mass. Since the three different accumulation batches started with different absolute PHB concentrations, relative PHB concentrations with respect to the initial level are reported enabling a direct comparison of trends (Figure 6.3 and SI).

For the aerobic group, trends of PHA concentration (Figure 6.3a and SI) reflect the trends of f_{PHA} (Figure 6.2a). Over 2 weeks, the amount of the polymer decreased to negligible levels (<4.5% of initial PHA) irrespective of environmental conditions applied. Aerobically, PHA is expected to be hydrolysed into hydroxybutyrate and then converted to CO_2 (Jendrossek et al., 2002).

PHB levels under anaerobic conditions (Figure 6.3b and SI) exhibited an initial rapid decrease within the first few days. In control experiments, with PHB in water was incubated for more than 30 days, and no hydroxybutyrate and VFAs were measured. This confirmed that the PHB chemical degradation was slow. It also supports that the initial rapid PHB loss was due to biological activity. After the fast initial degradation, the polymer amount stabilised. Therefore, both PHB concentrations and NPB concentrations were relatively stable. Methanogenesis onset was observed for the cases of initial pH of 10 at 37 and 55 °C after about 8 days. With the onset of methanogenesis, PHB losses resumed. Exogenous PHB was readily degraded, and endogenous PHB remained stable for several days under anaerobic conditions, notwithstanding conditions that promoted an initial degree of polymer mobilization. Thus, at least a fraction of the PHB-rich biomass can remain robustly intact, and stored polymer contents can be preserved anaerobically. Exploration



(a)



(b)

Figure 6.3: The trends of PHB concentration (normalised to the initial concentration) for the experimental groups with an initial pH of 7 and incubated at different temperatures (25, 37 and 55 °C) aerobically (a) and anaerobically (b).

for mechanisms and methods for mitigation of the observed mobilization of PHA mass is part of the ongoing investigation.

A relative loss of primarily PHA suggests mobilization and release of soluble products from the PHA-containing cells. Losses over time during anaerobic incubation of both PHA and NPB suspended solids may suggest a lysis of some PHA-containing biomass. The activated sludge is biomass with diversity in populations of species of bacteria. Selective staining and microscopy methods have revealed different morphotypes of PHA storing microorganisms also expressing differences in cell rigidity Pei et al. (2022b). Not all cell types will be equally robust to environmental stress, and this is, therefore, a challenge for downstream processing of mixed culture biomass. Optimal conditions for polymer quality preservation and recovery for some morphotypes may result in undue losses or, conversely, reduced yields for recovery from other morphotypes.

The main fermentation products during anaerobic incubation were acetate, butyrate and propionate. sCOD concentrations increased in correspondence to the removal of PHB and NPB suspended solids. PHA degradation is understood to follow steps starting with depolymerase activity releasing hydroxybutyrate. However, no build-up of hydroxybutyrate was measured, only volatile fatty acid formation. Soluble hydrolysis products may be released if host cells hydrolyze an excess of PHA and cannot metabolize it further under anaerobic conditions. These soluble products are readily fermented into acetate and butyrate. The theoretical levels of acetate and butyrate formation were estimated based on loss of PHA and the expected stoichiometry $2CH_3CHOHCH_2COO^- \rightarrow 2CH_3COO^- + CH_3(CH_2)_2COO^- + H^+$ for the extracellular depolymerase activity as reported by Stieb et al. (1984). The predicted levels matched well with the measured acetate and butyrate concentrations (SI). These results indicate that acetate levels were due to both PHA and NPB fermentation during anaerobic incubations. Butyrate formation was due to PHA fermentation, and propionate came from NPB fermentation. In the control incubation experiments, the exogenous supplied PHB formed acetate and butyrate following the stoichiometry reported by Stieb et al. (1984). Thus, the fermentation products during PHA-rich biomass anaerobic incubation experiments indicated that mobilized PHA becomes released to the matrix and is rapidly fermented. VFA formation lowers the system pH, and it is not clear in this work if the shift in pH and/or the build-up of VFA concentrations acted to inhibit further PHA mobilization. In another control experiment where PHB powder was incubated with commercially available lipase enzyme, an increasing level of HB was observed (SI). This suggests that the extracellular PHB hydrolysis can be carried out by a generic enzyme that could react to ester bonds.

Anaerobic conditions naturally preserved the accumulated PHA for a period of time better than aerobic conditions. However, even in the best case scenario (initial pH 7 and 25 °C), around 42% of the polymer was lost within the experimental period. Therefore, for temporary staging with storage or transportation of the PHA-rich biomass, anaerobic conditions alone are not sufficient, and results support that lower temperatures or pH levels need to be applied shortly after the accumulation process is terminated. In previous work up to pilot scale (Werker et al., 2020), PHA-rich biomass acidification down to pH 2 has been applied to induce higher polymer thermal stability before final dewatering and drying. However, even for this lower pH acidification treatment and wet biomass storage at 4°C, molecular weight loss still occurred at a quite slow but measurable rate. Selective staining with confocal microscopy evaluation (Pei et al., 2022b) of PHA-rich biomass incubated anaerobically with initial pH of 5.5 at 55 °C over 14 days revealed a dominance of intact cell structures containing PHA granules (Figure 6.4). Therefore, preserved PHA presented as still being intracellular after the anaerobic incubation (Pei et al., 2022b).

Neglecting the initial anaerobic PHA mobilization, intracellular PHA preservation was found to be possible due to a lack of oxygen supply. Intracellular depolymerase activity levels were influenced by pH and temperature but not prevented when oxygen was supplied. It has also been reported that environmental conditions can cause PHA granules to coalesce and become disrupted as functioning carbonosomes (Sedlacek et al., 2019b). The crystallisation of the polymer is reported to ensue upon dehydration, but before that, the polymer in a hydrated amorphous structure may form crystalline associations like a gel which could impede the intracellular depolymerase activity (Obruca et al., 2017; M. Porter et al., 2011). Meanwhile, the granules within intact cells may remain, kept away from extracellular depolymerase enzymes, which were anyway found to be inhibited by acidic conditions.

6.3.2. Incubation and Changes to Accumulated PHB Extractability

The observed trends illustrated in Figure 6.3b could be a mixture of effects, including discussed changes in granule structure or function, *in-situ* polymer crystallisation, depolymerase activity attenuation with time, and cell function losses due to prolonged anaerobic incubation. Increased temperatures to the thermophilic range are reported to induce changes in polymer morphology but will also increase depolymerase activity levels. Effects of pH on inferred in-situ PHA crystallisation have not been conclusive (M. Porter et al., 2011; M. M. Porter et al., 2011). A higher crystallisation rate with alkaline conditions than neutral and acidic pH has been reported, but results of opposite trends

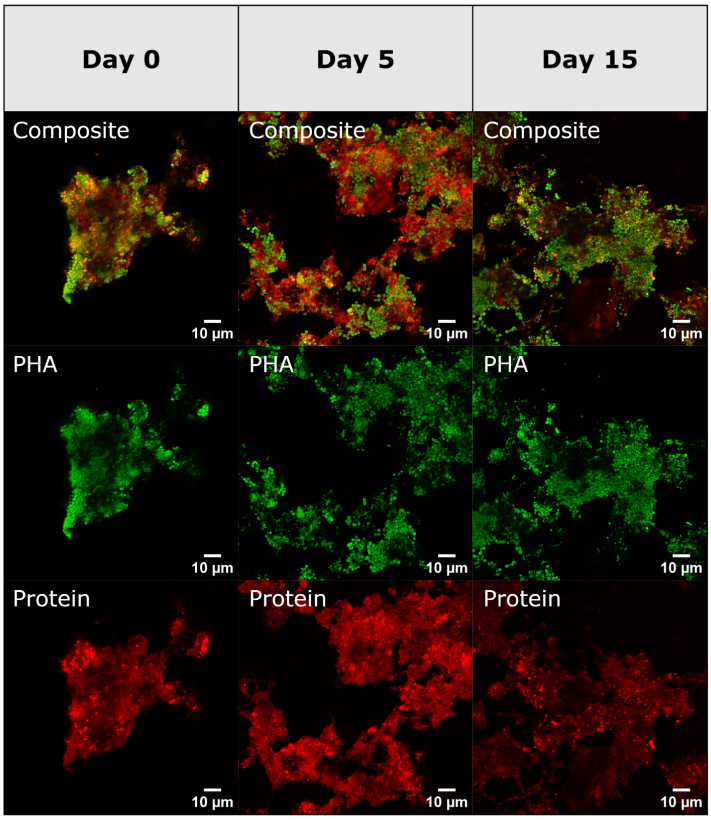


Figure 6.4: The selective staining of PHA (green) and non-PHA biomass (green) for one of the experimental groups under the anaerobic condition with initial pH of 5.5 at 55 °C.

with pH are also published (M. M. Porter et al., 2011; Sedlacek et al., 2019a). It is not a trivial matter to measure for changes in the polymer PHA nature *in-situ* without altering the polymer as a consequence of the measurement approach. Exploration of approaches is part of the ongoing investigation. The present study processed biomass samples using a standardized acidic wash and thermal drying protocol. Even if sample processing influences the polymer nature, it was interesting to observe if the incubation with different applied environmental conditions would result in systematic trends of polymer quality in the biomass.

Differences in polymer quality due to the incubation were assessed based on thermal decomposition temperature (T_d), polymer melt enthalpy (ΔH_m), and extractability. Thermal properties were assessed for the polymer in the dried biomass and for the extracted polymer. An advantage of PHB for the present investigation is that the homopolymer normally exhibits a relatively high crystallinity and crystallisation rate. This behaviour enables the evaluation of melt and crystallisation behaviour of the polymer even in dried biomass samples. Results are summarized in Table 6.4.

Acidic washing of the PHA-rich biomass is a method to enhance thermal stability (Chan et al., 2017). The (T_d) for the PHB in the biomass was consistent at 286±2 °C. Incubation did not influence the ability to harmonise for similarly enhanced polymer thermal stability before solvent extraction. Neglecting a measurement outlier (pH 7 and 55 °C), extraction resulted in recovered PHB with higher average thermal stability 297±1 °C as is expected. NPB has been shown to influence polymer thermal stability (Kopinke et al., 1996).

PHB melt peaks and enthalpies were quantified for the second heating ramp after a first melt and quench at −10 °C/min cycle to standardize the sample thermal history. Typical examples of the melting curve measured by DSC for the PHB standard, PHB in biomass and PHB films are shown in Figure 6.5. The melt peak temperature was estimated (T_m), and the peak melting enthalpy was referenced to the amount of PHB in the weighed sample. The extracted film T_m values (T_m = 170±4 °C) were on average comparable to the pure reference PHB (T_m = 170 °C). The melting temperatures for the polymer in the biomass were also similarly consistent (T_m = 175±3 °C), but on average 5 °C significantly higher based on a pairwise t-test (P=0.0002). This difference can be an artefact of differences in heat transfer properties (film versus powdered PHA-rich biomass), resulting in a lag in heat flux to and from the polymer in the biomass (Menczel et al., 2008). Therefore, different incubation conditions were not found to influence the polymer melting temperatures as expressed in the biomass in any systematic way and with respect to the standardized sample treatment protocol.

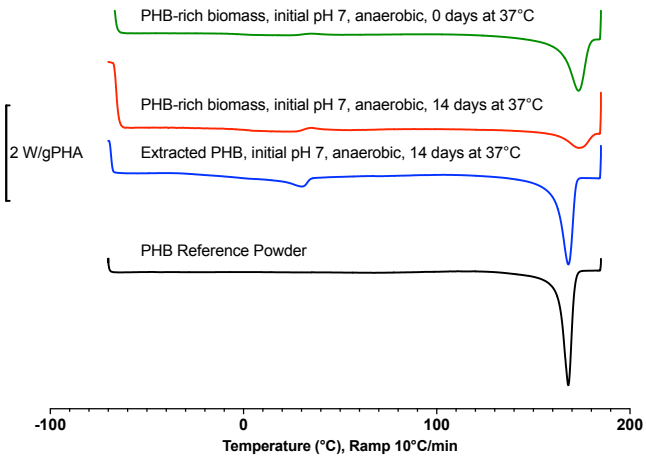


Figure 6.5: Melting of the standard PHB, PHB-rich biomass, and extracted PHB film measured by DSC.

The polymer melt enthalpy in the biomass was on average lower and more variable than for the corresponding extracted polymer (ΔH_m= 49±22 J/gPHA for PHB in biomass versus ΔH_m = 90±8 J/gPHA for extracted PHB). Variability of the extracted PHB melt enthalpy was not correlated to the recovered PHB purity, which ranged from 42 to 94 wt percent PHB. The reference PHB expressed a melting enthalpy of 98 J/gPHA. Melting temperatures and melting enthalpy depend on the crystalline lamellae thickness. This thickness varies with crystallisation conditions (Laycock et al., 2013). Differences in sample molecular weight, molecular weight distribution, and certain kinds of impurities may have contributed to the recovered polymer melt enthalpy variability and the value on average. These details for the extracted polymer require further investigation beyond the scope of the present study.

In contrast, the variability of the expressed PHB melt enthalpy for the polymer in the biomass could be coupled and correlated to the incubation conditions. In general, higher PHB-in-biomass enthalpies were found given shorter incubation times, lower incubation temperatures, or lower incubation pH conditions (Table 6.4). The melt enthalpy of semi-crystalline polymers relates to the degree of crystallinity with respect to the thermal history of the sample. PHB is expected to be able to reach a crystallinity of 60 percent (Laycock et al., 2013). While the direct estimation of polymer crystallinity from melt enthalpy measurements cannot be made with certainty, relative differences are indicative. Incubation time, higher pH, or higher temperatures reduced the ability of the polymer

mass distributed in granules within the dried biomass to crystallize.

Table 6.4: The properties of PHB standard, PHB in the biomass, and extracted PHB.

pH	T (°C)	Time	Extraction Efficiency (%)	gPHA/gTS		T_d		T_m		ΔH_m (J/gPHA)	
				Biomass	Film	Biomass	Film	Biomass	Film	Biomass	Film
PHB[1]	-	-	-	>99		298		170		98	
5.5	-	0	96	0.48	0.94	287	295	177	176	82	96
5.5	25	end	85	0.25	0.79	288	298	169	171	56	80
5.5	37	end	69	0.32	0.77	287	298	172	169	50	94
5.5	55	end	80	0.44	0.86	287	298	172	168	62	96
7	-	0	100	0.33	0.90	290	297	174	173	68	83
7	25	end	81	0.28	0.85	287	298	173	169	48	72
7	37	end	43	0.15	0.54	285	296	169	168	21	97
7	55	end	69	0.20	0.68	285	285	174	169	32	94
10	-	0	71	0.48	0.92	287	298	171	178	59	92
10	25	end	108[2]	0.18	0.75	288	299	173	169	66	92
10	37	end	65	0.08	0.46	284	296	178	166	27	101
10	55	end	52	0.08	0.42	283	295	170	168	11	89

[1] PHB standard acquired from Biomer with a purity >98%.
[2] Outlier.

A high relative crystallinity for PHB compared to the random co-polymer of poly(3-hydroxybutyrate-co-3-hydroxyvalerate), makes it a more challenging polymer to extract, especially when using so-called PHA-poor solvents (Werker et al., 2020). Longer extraction times or higher extraction temperatures are necessary to reach high extraction yields for PHB with all other things being equal. From the extraction mass balance evaluations, an average 77±19 percent of PHB was extractable from the PHA-rich biomass samples. Of notable interest from multilinear regression analysis of the extraction data (SI) was the finding that variability in extractability was positively correlated to the measured PHB-in-biomass melt enthalpy (P=0.0023). Therefore, the greater the expressed potential for the PHB in the biomass to crystallize, the better was the PHB extractability. The sampling protocol, involving an acid wash, thermal drying, and a melt and quench cycle before the melt enthalpy evaluation cannot represent the property of the PHB *in-situ*. However, the standardized sample protocol was still permitted to observe and measure for a change in polymer quality that had a direct bearing on the polymer extractability. These results suggest that recovery yields in the DSP are influenced by the condition of the polymer in the biomass, which can be affected by the applied methods of recovery. pH condition influenced the polymer condition from the start. An acidic pretreatment was found to be preferred for improved extractability.

The recovered polymer was not pure, and because the solvent was evaporated from the extracted solids, the solubilized NPB was also evaluated. On average, 8±2 percent of the NPB was co-extracted with the recovered polymer. Therefore, biomass with lower PHB

content yielded an extracted polymer of lower purity for the total extracted solids. Multi-linear regression analysis suggested that less NPB was extracted given lower temperature incubations (P = 0.0367). Higher incubation temperatures may generate solvent soluble NPB. PHA-rich biomass pretreatment rather than post treatment extracted polymer washing to remove unwanted extraction impurities requires further systematic fundamental and techno-economic evaluation to improve DSP process and economy.

6.4. Conclusions

- Intracellular depolymerase activity for a mixed culture PHA-rich biomass can be constrained by anaerobic conditions but changes in environmental conditions may nevertheless cause significant polymer mobilization even for short term storage (up to 48 hours).
- Extracellular depolymerase activity for a mixed culture PHA-rich biomass is limited for an extended period of time so long as the PHA remains intracellular.
- Acid environmental conditions are preferred to preserve quantity and quality for recovery of PHB from PHA-rich biomass following a mixed culture accumulation process.
- The melting enthalpy of PHB in the biomass following a standardized sample workup can be indicative of PHB extractability from biomass.

6.5. Acknowledgement

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Supplementary Information

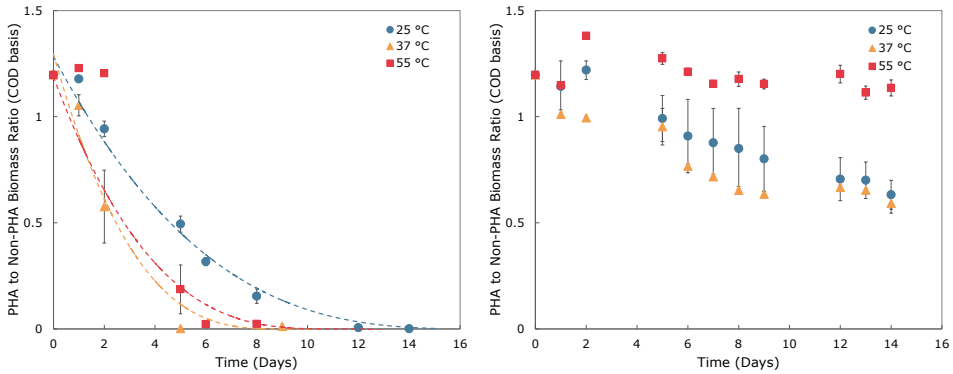

Figure S1: The PHA to non-PHA biomass ratio (f_{PHA}) over time for the experimental groups with an initial pH of 5.5 and incubated at different temperatures (25, 37 and 55 °C) aerobically (left) and anaerobically (right).

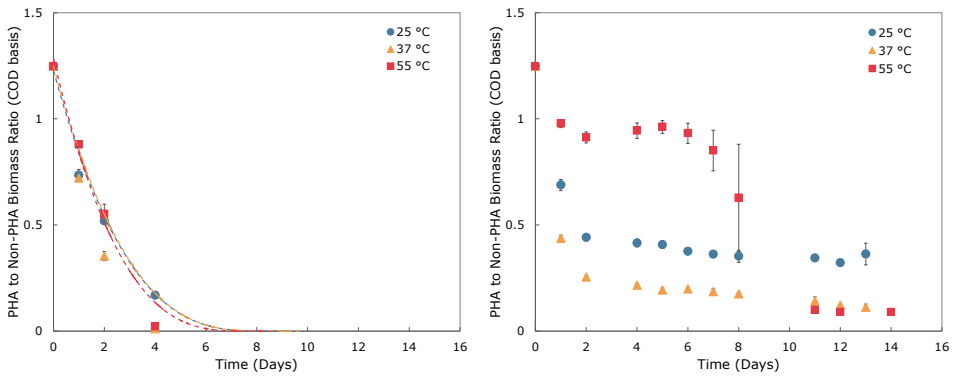

Figure S2: The PHA to non-PHA biomass ratio (f_{PHA}) over time for the experimental groups with an initial pH of 10 and incubated at different temperatures (25, 37 and 55 °C) aerobically (left) and anaerobically (right).

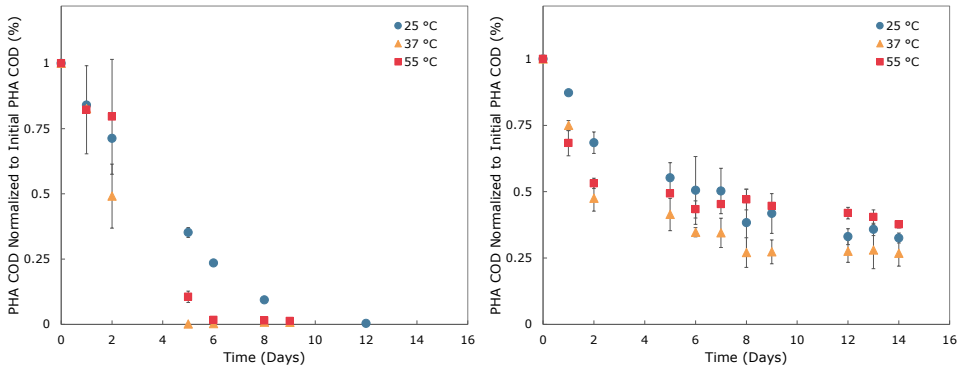

Figure S3: The trends of PHA concentration (normalised to the initial concentration) for the experimental groups with an initial pH of 5.5 and incubated at different temperatures (25, 37 and 55 °C) aerobically (left) and anaerobically (right).

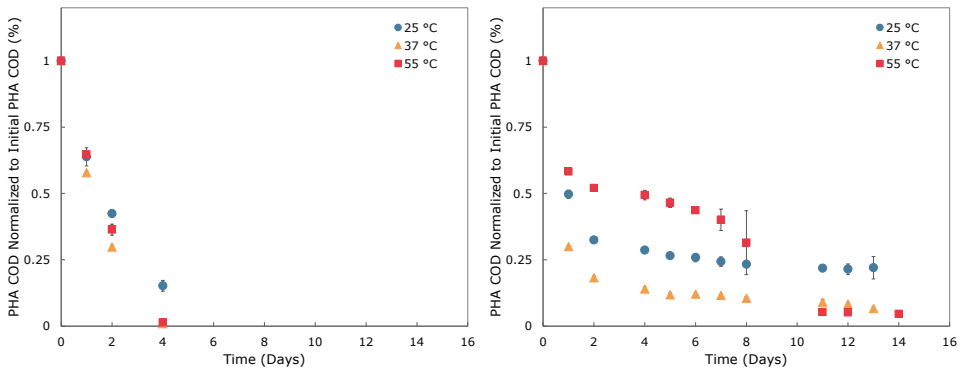

Figure S4: The trends of PHA concentration (normalised to the initial concentration) for the experimental groups with an initial pH of 10 and incubated at different temperatures (25, 37 and 55 °C) aerobically (left) and anaerobically (right).

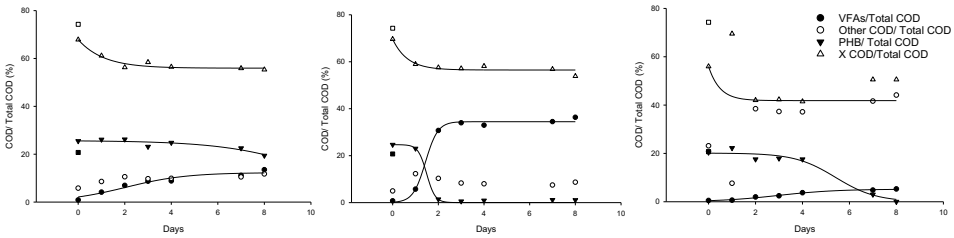

Figure S5: The trends of PHA concentration, non-PHA biomass concentration and VFAs concentration on a COD basis (normalised to the initial total) for the control experiment at Initial pH 5.5 (left), 7 (middle) and 10 (right). In the control experiment, exogenous PHB was incubated together with activated sludge and the sludge from an anaerobic digestor. The control experiment was carried out anaerobically at 37 °C.

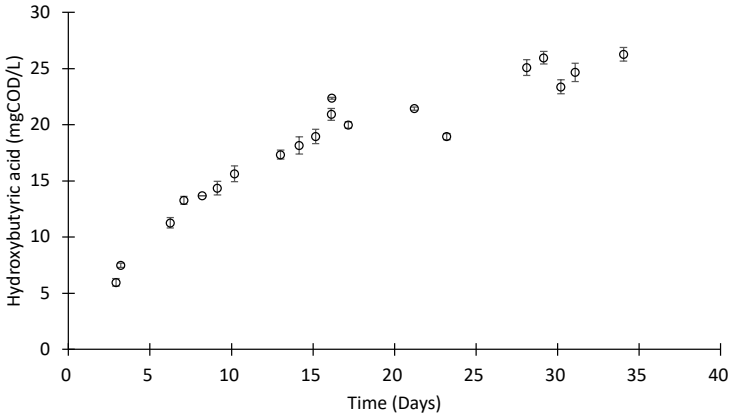

Figure S6: The trends of HB concentration in the control experiment, where exogenous PHB was incubated together with Lipozyme® CALB (Novozymes, Denmark) from *Candida Antartica* B.

Table S1: The loss of PHB COD (before the on-site methanogenesis activity) and the estimated acetate and butyrate based on the stoichiometry $2CH_3CHOHCH_2COO^- \rightarrow 2CH_3COO^- + CH_3(CH_2)_2COO^- + H^+$ reported by Stieb et al. (1984).

mg COD	Initial pH 5.5			Initial pH 7			Initial pH 10		
	25 °C	37 °C	55 °C	25 °C	37 °C	55 °C	25 °C	37 °C	55 °C
Loss of PHB	2220	2488	1641	1414	1785	2600	2743	3264	1972
Theoretical butyrate	1013	1135	749	645	814	1186	1251	1489	900
Measured butyrate	1104	1160	770	675	870	1303	1263	1490	909
Theoretical acetate	734	823	543	468	591	789	942	1080	653
Measured acetate	1295	1295	883	1101	1241	1733	1557	1737	1191

Table S2: Detailed description of the PHB extraction.

pH_i	T (°C)	Time	Input Suspended Solids Concentration			Extracted Soluble Solids		
			X_{PHB} mgPHB/gDMC	X_{NPB} mgPHB/gDMC	ΔH_{mx} J/gPHA	S_{PHB} mgPHB/gDMC	S_{NPB} mgPHB/gDMC	ΔH_{ms} J/gPHA
-	-	0	21.88	19.84	82	20.95	1.35	96
5.5	25	end	9.91	25.84	56	8.39	2.20	80
5.5	37	end	20.35	37.48	50	14.11	4.14	94
5.5	55	end	27.35	29.60	62	21.99	3.48	96
-	-	0	17.45	28.31	68	17.45	1.92	83
7	25	end	27.61	63.41	48	22.28	4.07	72
7	37	end	16.99	82.77	21	7.30	6.18	97
7	55	end	24.38	83.30	32	16.71	7.73	93
-	-	0	41.70	38.06	59	29.71	2.70	92
10	25	end	13.03	53.95	66	14.13	4.82	91
10	37	end	4.05	41.89	26	2.65	3.08	101
10	55	end	2.33	21.12	11	1.24	1.68	89

7

Outlook

Efforts to motivate industrial implementation of open culture PHA production processes have promoted ongoing research and development journey over the past 20 years (Á. Estévez-Alonso et al., 2021a; Kourmentza et al., 2017). This thesis investigation has focused on advancing potential for PHA production processes that directly use municipal activated sludge as the accumulating biomass. While undertaking the thesis research, a focus has been on principles and fundamental understanding that could further help to facilitate success in the ongoing up-scaling efforts with supporting industrial, water authorities and private sector stakeholders.

The thesis research contribution to the efforts in the up-scaling journey started with a critical review (Chapter 2) of the milestones that have been reached with PHA accumulation processes now more frequently undertaken at pilot scale. Potential bottlenecks to progress were identified as knowledge gaps underlying work reported in the following research chapters. Chapters 3 and 4 work directed to developing visualisation (Chapter 3) and quantification (Chapter 4) methods through staining and microscopy that could be used to better understand the distribution and degree of enrichment of PHA-accumulating microbes in a municipal activated sludge. Bath wastewater treatment plant (WWTP) was used for a case study. Results showed that 48% of the microbial biomass was engaged with PHA accumulation, and this was defined as the degree of enrichment. Furthermore, 22% of the microbial biomass was active but did not accumulate PHA, and the remaining 30% of this biomass was inactive and assumed to be inert organic matter. The 22% flanking active microbial mass will contribute to oxygen consumption and compete for substrate during the PHA accumulation process. The combination of 22% flanking population and 30% inert organic solids also mean a lower average biomass PHA content by the end of the accumulation process. Since only around half of the activated sludge was accumulating PHA, the PHA accumulation capacity for the PHA-storing microbial biomass was estimated to be higher on average (0.64 gPHA/gVSS) than measured for biomass PHA content as a whole (0.50 gPHA/gVSS). A practical limit for PHA production in a municipal activated sludge has not been established previously, and this set a benchmark for process optimisation strategies.

The established staining methods were then applied (Chapter 5) to activated sludge collected from 6 different WWTPs to understand similarities or differences of experience compared to the Bath WWTP case study. The results showed that, for municipal activated sludge, the degree of enrichment determined the biomass PHA accumulation potential. Furthermore, the average PHA accumulation capacity for the accumulating fraction of the biomass gave similar results to Bath WWTP (0.58 ± 0.07 gPHA/gVSS). These findings

revealed the upper limits of the biomass PHA content for the PHA accumulation process directly using waste municipal activated sludge produced in a Northern European climate. It also indicated that increasing the degree of enrichment, in the WWTP or in the PHA accumulation process, is the route to reach maximum PHA accumulation potential.

The staining methods applied to a specific activated sludge from Bath WWTP in Chapter 3 revealed a diverse microbial community. For Bath WWTP, two groups of PHA-accumulating microbes were found. One group of PHA-storing microorganisms was observed to be "stretchy" due to increase in cell size during accumulating PHA granules. The other group of microorganisms were stiff due to the observed cell size remaining similar before and after PHA granule accumulation. The distinct differences among different cell types implicated challenges for the post accumulation downstream processing (DSP). If the effect of different DSP steps influence different fractions of the PHA-containing biomass to different extent, then optimal conditions may be difficult to find without losses or degradation of polymer in the process.

After the PHA accumulation process, steps of DSP are applied to stabilize, purify and recovery the polymer from the rest of the biomass. When accumulation substrate feeding and oxygen supplies stops, the stored PHA-granules in microbes have an inherent ecological survival role as an endogenous carbon and energy reservoir. Any microbial activity on the intracellular stored polymer granules leads to anticipated changes in polymer quantity as well as polymer properties. In Chapter 6, the fate of exogenous and endogenous PHA under different environmental conditions for an activated sludge was studied. An influence of temperature and initial pH were examined. The results confirm that anaerobic acidic environments are more conducive for preserving the PHA quantity and quality. However, it was also revealed that a significant fraction of the PHA in the biomass is susceptible to become mobilized and fermented if optimal conditions of preservation are not applied quickly after the accumulation process is stopped.

Building on knowledge from the previous research, the new findings and insights found as part of this thesis work suggest opportunities and knowledge gaps to be tackled with ongoing efforts to reach successful industrial processes. Identified challenges and research considerations for future developments are presented in the following three sections. The principal goals with continued research and development include driving the selection of PHA-accumulating microbes in WWTPs to reach a higher degree of enrichment in waste activated sludge; manipulating the activity of the microorganisms during PHA accumulation processes for selective growth of PHA-accumulating microbes; steering and preserving polymer quantity and quality during the PHA production, and especially

during DSP; and setting the landscape for up-scaling PHA production with activated sludge.

7.1. Selection of PHA-accumulating Microbes and the PHA Accumulation Process

A municipal WWTP entails inherently dynamic environments due to the process hydraulic flow pattern (plug flow) with recirculation and/or changes in diurnal load. Such dynamic environmental conditions can generate a periodic exposure of biomass to feast-famine regimes and this can be selective towards growth of PHA accumulating microorganisms in the biomass. Chapters 3 and 4 suggest that selection of PHA-accumulating microbes has at least two aspects. The first is the resulting degree of enrichment for the biomass, and the second is PHA accumulation capacity of those selected PHA-accumulating microbes. Chapter 5 reports on varying degree of enrichment between six WWTPs but findings also suggested for a consistency in the average PHA accumulation capacity for the PHA-accumulating fraction. This outcome raised three questions:

1. What determined the degree of enrichment between the WWTPs?
2. Why did the selected PHA-accumulating microbes exhibit similar PHA accumulation capacity regardless type of WWTP and degree of enrichment?
3. Why is the PHA accumulation capacity in tested WWTPs, and in enrichment cultures in general, lower compared to the experience of *Plasticicumulans acidivorans* enriched at lab scale (Johnson et al., 2009)?

Chapters 3, 4 and 5 report on an active flanking population that was not seen to be accumulating PHA. Logically, one would like to minimise activity of such flanking populations and to increase the degree of enrichment for higher biomass PHA contents and higher productivity in a PHA-accumulating process. However, it is vital that increase of degree of enrichment sustains function of the biomass in activated sludge. It is necessary to sustain presence of any flanking populations if their activity is critical for pollutant removal during the process (i.e. nitrification, denitrification, phosphorus removal, etc.). Otherwise, if PHA accumulating microorganisms could carry functions of treatment for the organisms they replace then, it is worth to drive the process for increasing degree of enrichment. All optimisation strategies for degree of enrichment in the WWTP need to ensure that

core pollutants removal function of WWTP are not compromised. Research approach of assessment of process function with modern molecular microbiology methods like sequencing, Fluorescence *in situ* hybridization (FISH), and isolation of PHA-accumulating microbes in activated sludge can help to relate outcomes of anticipated redundancy in community structure function with wastewater treatment, given process engineering for improved degree of enrichment.

If it is feasible to minimise the flanking population already in the waste activated sludge, one can start to better tune the biomass characteristics for PHA production. Since it was found in this thesis work that only a fraction of the activated sludge was accumulating PHA one can consider ways to increase selection pressure for PHA-accumulating microorganisms. Even though there must be some selection pressure in general because of feast-famine regime, more detailed analysis is needed to gain further insight to confirm the sources of selection pressure. As the first step, the existing nature of a feast-famine regime in the WWTPs need to be understood and somehow quantified. One possible approach is to sample the WWTP at different locations in the treatment train and at different times during the day. Chemical Oxygen Demand (COD) including readily and more slowly biodegradable fractions, volatile fatty acids (VFAs), and nutrient (phosphorus and nitrogen) removal and biomass activity trends could be characterized and subsequently modelled. The modelling could evaluate for competition between PHA storing and non-storing heterotrophic metabolism. Biomass PHA content and selective staining could also be applied to calibrate the process model for degree of enrichment. Process modelling of optimisation to increase selection pressure could be tested first via simulations, and then with practical evaluations. Directions for proven engineering of WWTP configurations to get higher degree of enrichment can be applied, and consequently higher biomass PHA contents can be achieved. Activated sludge can then become a generically attractive source of biomass for industrial scale PHA production.

For activated sludge, as shown in Chapters 4 and 5, PHA accumulators were selected, but the PHA accumulation capacity for the selected PHA-accumulating microbes was lower than the extreme level of 90% gPHA/gVSS of *plasticicumulans acidivorans* (Johnson et al., 2009). These results, and results of the literature in general, suggest that engineered environments can be selective towards PHA-accumulating microorganisms. But the engineered environments are not necessarily selective towards microorganisms having extreme PHA accumulation capacity. At the same time, *Plasticicumulans acidivorans* was enriched from municipal activated sludge as the inoculum. The average biomass PHA content for the accumulation capacity was estimated with the developed staining method

but the distribution of accumulation capacity in each types of cells is still unknown. For the PHA accumulation fraction, Chapter 3 was intriguing because there were at least two distinct groups of PHA storing microorganisms. For one group, the cell size increased during PHA accumulation. For the other group, the cell size remained similar before and after PHA accumulation. This stiff cell wall group may be expected to be physically constrained in biovolume and accumulate less PHA than the stretchy group. If this is the case, then the distribution of PHA capacity in the biomass is likely to be biomodal. What distinguishes between selection of "stiff" and "stretch" cell types need to be elucidated in future research. These observations were made for Bath WWTP in a first case study. It is necessary to evaluate more WWTPs before any general conclusions may be drawn. In the meantime, one may also apply a bottom-up microbial ecology approach for a more detailed community analysis. A traditional method could be used to work isolate/enrich for stretchy and rigid cells separately through contrasting selection including methods known to work for hyper-accumulating microorganisms. Then it is a case to study and compare individual physiology and PHA accumulation potential. The developed modern molecular microbiology methods also can permit that a complex biomass can be dissected and assessed. Raman image-assisted or fluorescence-activated cell sorting could be used to sort different PHA-accumulating microorganisms with a high throughput. Once the cells are sorted, the cell physiologies could be analysed and compared even at the single cell level. These methods may elucidate if interpreted differences between the stretchy and stiff PHA accumulators give meaningful clues to distinguish between selection for the PHA storing phenotype and selection for the phenotpye with high accumulation capacity.

For the thesis work, activated sludge was directly used for PHA accumulation. The accumulation was started with an acclimation protocol by disposing the biomass to three feast-famine cycles. Previous research has found that acclimation results in a higher biomass PHA content for activated sludge biomass (Morgan-Sagastume et al., 2017). The underlying mechanisms for improvement due to acclimation are still not clear. In Chapters 3 and 4, 16S rRNA was quantified during the acclimation and accumulation phases as an indicator for cellular activity. However, no significant differences were found regarding the general biomass activity levels. Therefore, acclimation does not seem to be a kind of "wake up call" for the biomass. An effect of acclimation can be that the existing PHA-storing fraction of the biomass store more PHA during the accumulation process. It can also be that the PHA-storing fraction is stimulated to grow more during the PHA accumulation process. The methods of staining can be applied to contrast the expressed degree of enrichment for accumulations with and without an applied

acclimation. More specific methods for quantifying PHA accumulation activity could also be applied in future research. For example, selective staining methods such as mRNA-FISH targeting at PhaC gene (responsible for PHA synthesis) and BONCAT FISH may help for understanding the general protein synthesis activity. Methods like proteomics could also be applied to quantify the change of specific proteins of interest during the acclimation and accumulation processes.

In a parallel thesis work at Wetsus, A. Estevez-Alonso observed that it was possible to drive a stable PHA accumulation process with biomass growth and sustained PHA content for up to 72 h (data not shown). This outcome suggested that even if the current PHA accumulation methods work well, there is still room for improvement. The process could be further optimised to increase the long-term stability and better promote for selective growth of the PHA storing biomass to increase the degree of enrichment during the accumulation process. The research of Ajao (2020) showed also that in a process with nutrient limitation, extracellular polymeric substances (EPS) were produced instead of PHA. Understanding the competition between EPS and PHA production could help develop the opportunity to switch or even combine the production of EPS and PHA in an engineered waste activated sludge post production process after wastewater treatment.

7.2. Polymer Properties and Downstream Processing

PHAs are a family of polymers. Different types of PHAs can exhibit very different kinds of chemical, thermal and mechanical properties. Moreover, the polymers recovered from a biomass are most often co-polymer blends. In the present work, acetic and propionic acids were used with activated sludge to produce poly(3-hydroxybutyrate-co-3-hyroxyvalerate) or PHBV. Properties will be influenced by such things as molecular weight distribution and as well as the 3-hydroxybutyrate to 3-hydroxyvalerate ratio. Properties of molecular weight and composition distributions influence melt and crystallisation behaviour. These properties affect the DSP conditions for polymer recovery, and the range of possibilities of the polymer in potential for applications. Current research literature indicate that if the biomass PHA properties before extraction are known, the properties of the extracted PHA can be predicted (Bengsston et al., 2017; Á. Estévez-Alonso et al., 2021a). Properties differences are expected to lead to different requirements in the optimal DSP conditions. It is critical to understand how properties are influenced during the DSP and to confirm if optimal conditions are just dependent on the polymer type and/or the biomass source as well. Therefore, a detailed research is needed by sampling during

the accumulation process and assessing the PHA properties. An experiment similar to Chapter 5, but focusing on PHA properties, could be performed. This understanding will help future up-scaling processes regarding the process applicability, management of the PHA properties, and product quality control.

After PHA is accumulated in the activated sludge, DSP steps are needed to recover the intracellular PHA for further use in applications. As identified in Chapter 2, DSP is one research bottlenecks for up-scaling. DSP is an involved series of process steps including conditioning the PHA-rich biomass, drying, PHA extraction, and further purification if necessary. In recent years, research attention has shifted to include DSP. These research efforts have primarily focused on extraction methods such as water or solvent based methods. Most work has only included outcomes of recovery efficiency and product purity but less attention has been given to other polymer properties. Most of the extraction work is performed at the lab scale, where only a small amount of PHA-rich biomass needed to be processed. Often the starting material for evaluations is a freeze-dried PHA-rich biomass collected after one or several accumulation experiments. Meanwhile, the effects of conditioning the PHA-rich biomass may have been overlooked. The conditioning methods, and time scale in application of the process steps has been shown to be critical for full-scale PHA production process in terms of logistics and needs for temporary storage and/or transportation. In Chapter 6, effects of different environmental conditions on the fate of the PHA in activated sludge were evaluated. It was found that acidic conditions favor the preservation of the polymer. However, a more detailed evaluation of properties and mechanistic explanation for the initial rapid mobilization of a significant fraction of PHA even under anaerobic conditions is needed.

As shown in Figure 7.1, while conditioning the PHA-rich biomass, the responses of PHA-rich biomass are expected to be influenced by interlinking factors. During the conditioning, PHA depolymerase activity is a first main concern. Once the environmental factors such as oxygen level, temperature, pH, and ionic strength change as part of the conditioning, they may be influenced by the integrity of function of the PHA granules. Changes in the intracellular polymer condition may be influenced by molecular weight, monomer composition as this can influence the driving forces for changes in crystallinity. Enzyme activity and the ability of the enzyme to interact with the polymer due to any onset of crystallization are relevant to understand and engineer in process. Interaction between the PHA and the intracellular enzymes will result in degradation of molecular weight and mobilization and release of soluble products as interpreted in the present work. The effects of the environmental factors on PHA properties are likely to be dynamic. In the present

work changes were interpreted due to the effect of anaerobic incubation on the changes in polymer melt enthalpy following a standardized protocol. These changes furthermore corresponded to a decrease in the extractability of the polymer from the biomass. A challenge is to measure or interpret to understand changes occurring for the polymer directly for the granule *in-situ*. Sampling handling for measuring polymer properties also inherently creates environments that can influence the polymer properties.

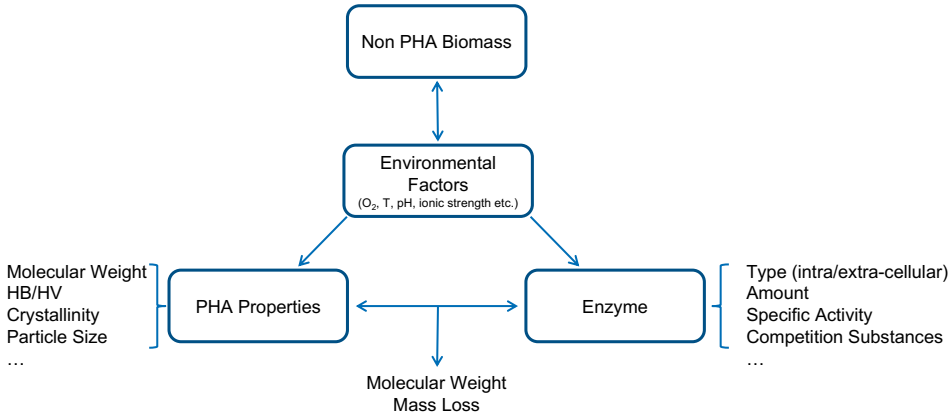

Figure 7.1: The interactions among the PHA, environmental factors and depolymerase enzyme

The two main challenges for differentiating the effects described in Figure 7.1, are the complexity of the PHA-rich biomass, and the PHA properties measurements. The complexity of the PHA-rich biomass is challenging because of the large fraction of non-PHA biomass (50% of VSS) and the presence of different PHA accumulating microorganisms. A simplified experimental system using enrichment or pure culture is preferred to establish principles that can then be validated as observations for more complex biomass like activated sludge. Enrichment or pure cultures will typically have a higher biomass PHA content, and only one dominant species of PHA-storing microorganisms. If different types of PHA-accumulating microbes could be separated as discussed in Section 7.1, then one could also test the responses of these separated microbes to the different environmental conditions.

The PHA properties measurements are challenging because the current analytical methods do not analyse PHA in its native environment and the complexity due to a heterogeneity of types of PHA-accumulating microbes. The PHA is accumulated as an intracellular granule referred to as the native polymer. The native PHA granule is in a special status and is considered to be hydrated, and as such, amorphous or mobile. Different environ-

mental conditions may disrupt the status of the PHA granules and cause a change in to crystallinity or other properties like cation association of the PHA which may result in different responses to depolymerase activity. PHA analytical methods of properties can require pH change for the PHA-rich biomass, thermal drying, and polymer extraction. This sample preparation will affect the properties of the native PHA granule; thus, the direct effects of the environmental factors cannot be measured. If the analytical methods still allow to observe for relative changes of a property then interpretations can still be made. However, an influence of the preparation method in of itself cannot necessarily be easily ruled out from such an interpretation.

In Chapter 6, a step was made to measure for a change in PHA properties directly in the biomass. The melting and crystallisation behaviour of accumulated polyhydroxybutyrate (PHB) was measured in dried biomass using Differential Scanning Calorimetry (DSC). The DSC applied directly to the PHA-rich biomass gave promising results for characterising the melting and crystallisation properties of PHB and showed for signs of an influence of different incubation conditions. X-ray diffraction analysis (XRD) can give more direct information about the crystallinity of the PHA and the crystalline structure of PHA without needing to melt the polymer. In a preliminary test to see if the PHB in the biomass could generate a specific signal, a PHB standard (>99% purity) and a thermal dried PHA-rich biomass from the PHARIO project were analysed using Bruker MeasSrv (D2-205787)/D2-205787 with Cu tube (30kV, 10mA) and LynxEye detector. The data were collected in the angle range $5° < 2\theta < 90°$ with a step size of 0.02° (2θ), and the total measuring time was around 2.5 h for each sample. The preliminary results (Figure 7.2) confirmed that the *in-situ* PHA in the biomass could generate a comparable signal pattern to the standard sample.

For future research, the sample preparation could be further optimised by freeze-drying fresh samples directly. This sample preparation is less likely to influence *in-situ* properties as thermal drying and thus keeps a possibility to maintain the intracellular PHA as close to its natural *in-situ* status as possible.

The heterogeneous microbes and the accumulated individual PHA granules also contributed to the complexity of the *in-situ* PHA properties measurement. Polymer extraction blends the properties of the individual PHA granules in the form a co-polymer mixture. Methods need to be developed to evaluate if the different groups of microbes accumulate different PHAs (molecular weight and monomer composition). Methods like Raman or Transmission Electron Microscopy could be implemented to test the degree to which accumulated PHA granules are heterogeneous. Alternatively, as described before, the

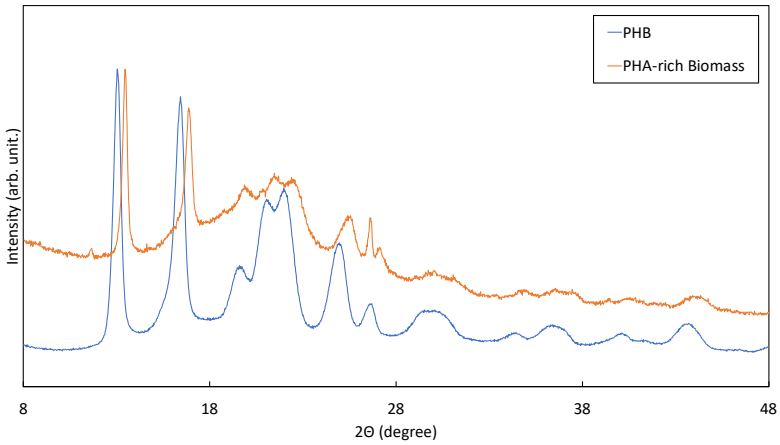

Figure 7.2: Preliminary X-ray diffraction analysis (XRD) results for PHA (>99% purity) and PHA-rich biomass from PHARIO. XRD was performed using Bruker MeasSrv (D2-205787)/D2-205787 with Cu tube (30kV, 10mA) and LynxEye detector. The data were collected in the angle range $5° < 2\theta < 90°$ with a step size of 0.02° (2θ), and the total measuring time was around 2.5 h for all samples.

separation proposed in Section 7.1 could be implemented then the properties of the microorganisms can be analysed in grouped sub-samples.

Implementing a simplified experimental system and developing *in-situ* analysis methods could differentiate the factors mentioned in Figure 7.1. Such developments can lead to insights that may help clarify underlying mechanisms the results and observations reported in Chapter 6. With improved insight the conditioning of the PHA-rich biomass could be further engineered and optimised.

7.3. Scaling-up and Beyond

Despite the range of hanging fundamental questions discussed so far, as related in Chapter 2, PHA production technology readiness level today can already facilitate industrial scale implementation. The lack of material for prototyping applications has hampered technology pull for up-scaling of the process. As illustrated in Figure 7.3, working with lab and pilot scales may produce in the order of 5 and 500 gPHA/d, respectively. These low amounts are a mismatch to the 10s of kilogram expectations from application development stakeholders. During course of this thesis work, we contributed know-how and participated hands-on a 4 m^3 accumulation production trial used for finalizing design

of a demonstration scale plant at 10 m^3. The demonstration scale production facility was inaugurated in May 2022. Such a demonstration plant is a technology enabler to connect to downstream stakeholders and establish industrial practice for the PHA production process. Production of larger amounts of material (Figure 7.3) for prototyping is essential to break the present-day catch-22 that hold back development of commercial supply chains.

Figure 7.3: The volume of the reactors at different scales and the estimated PHA productivity. Photos by Ángel Estévez-Alonso, Erik de Vries, Paques Biomaterials BV, PHA2USE in the order of appearance.

Steps to commercial scale PHA production must solve the critical logistics challenge. Where should the production site be? One of the possible schemes is to produce PHA at more central sludge processing facilities. Before transport to such a facility, the municipal waste activated sludge is dewatered. However, for PHA production, the thickened activated sludge needs to be diluted again. One may avoid dewatering and dilution by placing the PHA accumulation reactor beside the wastewater treatment plant.

Another challenge is where can realistic stable VFAs sources be secured from. One needs to find reliable VFAs sources, for example, the fermented primary sludge. More importantly, the scale of the source must match the designed PHA production capacity. However, since VFAs are platform chemicals that are used for other resource recovery purposes than for PHA production, competition exists. VFAs are central for biogas production and as chemical products from chain elongation. Competition due to demand for VFAs drives increased cost for production. This production cost must be met by the value of the product through its downstream application. So the overall economy can be quite specific to the polymer application and may need to be assessed and justified case by case. Therefore, a detailed economic and life cycle assessment is needed to support the

decision-making.

The residue of PHA-rich biomass after PHA extraction also needs to be valorised if at all possible. PHA production uses the waste activated sludge as a catalyst which means the sludge mass is not reduced. The residues of PHA-rich biomass after PHA extraction are dry solids still needs to be managed. One way to manage this by-product it is to generate syngas and use it also to produce PHA. The PHA production process approach a reach zero waste concept if this would be achieved. Next to the existing WWTP, new concepts of WWTP are developing, and new WWTPs are built over time. The Nereda® process, where aerobic granular sludge is one example. The advantages of the Nereda process include a small footprint, lower environmental impact, and lower cost. Additionally, resources like Kaumera Nereda® Gum and phosphorus can be recovered from the process and waste surplus biomass (Feng et al., 2021; Rodriguez Perez et al., 2018). In Chapter 5, we evaluated to accumulate PHA using waste Nereda® aerobic granular sludge (data not shown). The maximum biomass PHA content was not high at 10 percent. One of the reasons for the outcome could be that the accumulation process method was not ideal for this type of biomass. If the accumulation could be tuned, then the PHA production could be coupled into the resource recovery portfolio for Nereda® WWTP. Kaumera Gum is extracted under alkaline conditions, and as described in Chapter 6, alkaline conditions are detrimental to PHA quantity and quality. Therefore, if PHA production could be achieved using aerobic granular sludge, then a combined PHA and Kaumera Gum extraction would need to be designed and integrated.

To develop commercial production of PHA derived from waste activated sludge, other stakeholder aspects, such as regulation standards, as well as societal and business development perspectives, will need to be evolved and established. PHAs that are recovered and sold must be standards for specific kinds of applications as plastics and chemicals are regulated in general today. Even though PHAs represent ideals in development for a biobased society, that dream can only be realized with public education for awareness and safe use given responsible marketing and branding tools to anchor these advancements with benefits to society through successful products and business cases.

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Office is a place where I spend a lot of time (not working but talking). Thanks to all my officemates from three different offices. Thank you **Thomas, Victor, Catarina, Goncalo, Shuyana, Wiecher, Andrew, Swarupa, Daniele, Suyash, Antony, Xiaoxia, Carlo and Cristina**. **Thomas**, you are nice in your way. Thank you for all the 'shooting', activities you organized and the enthusiasm for the China trip. Hopefully, soon, I will visit you in France, and you will come to China with me. I also would like to thank you, **Rebeca, Maarteen and Michele** for participating in lots of sports evenings during COVID. **Goncalo and Shuyana**, you need to come too. **Goncalo**, it took time to make you more social. Still, I am glad we had many nice conversations as time passed. **Shuyana**, it was nice to sit with you for four whole years. It was great that you initiated many office activities! **Catarina**, thank you for trusting me to take care of your office plant :) **Antony**, we did not share the office for a long time, but you were an excellent disturbance. I appreciate your support when I was anxious about my future. **Carlo**, you are such a nice person, considerate, nice

to talk with, and always willing to help. Thank you for checking on my family and me when I was travelling and in China. Please be more successful with your music career so that I can be your full time manager! By the way, I still think you can be a vocalist, so you should sing more. **Xiaoxia**, it was a pleasure to join your wedding, to have dinners at your place and to have good conversations. Good luck with Wetsus China, you are doing great! **Cristina**, I knew you during my master study. As I always said, I enjoyed working with you and learned a lot. Both of us have strong personalities, and I think we managed well. I thank you for your support while I was searching for a PhD position. I am also grateful for making the joint effort with Pieter to keep me at Wetsus after my PhD. Hopefully, we can keep our collaboration in the future.

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have had at Wetsus.

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Chris, I still cannot believe your legal name is Christopher. It seems too long to combine with short, oops, no, Schott. You looked too serious when I met you, but soon I found that

we had compatible personalities. My primary social activities were the BBQ, the parties, the trips, and the conversations with you for a long time. It is not easy to make a genuine connection, but it would not fade away easily.

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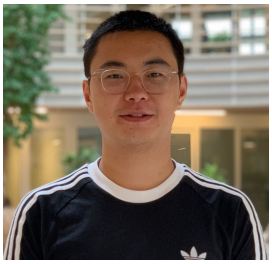
距离让维持友谊变得很困难。虽然我们很少联系，但每次回国见到你们，跟你们谈天说地还是感到十分亲切。我很感谢你们，萝卜，谢彩蛋，石头，吕泽智，刘培宇，宋梦芸，段永红。王周钊，感谢你不时的问候，你在上海多挣钱，我以后去给你开车。段巧，虽然我们不常见面，也过着不一样的生活，但还是很开心能时常跟你聊天。希望你早日来欧洲旅游！

我最亲爱的爸爸妈妈，谢谢你们这么多年来对我无条件的支持和鼓励。因为你们，我可以看到更大的世界。因为你们，我对生活充满了热爱。也因为你们是我坚强的后盾和温暖的港湾，所以我可以更勇敢更自由的去闯。人生有得必有失，在外多年你们少了我陪伴在身边的日子，但我想说我爱你们。姥姥，大伯，大妈，姑姑，姑父，叔叔，婶婶，大姨，小姨，姐姐，你们对我的关心不在言语里而在行动中。我尤其感谢你们在我不在的日子里，对我爸爸妈妈的陪伴和照顾。

Curriculum Vitæ



Ruizhe Pei

Ruizhe Pei was born on December 5th 1992 in Xinjiang Uygur Autonomous Region, the People's Republic of China. From 2010 to 2014, for his bachelor, he studied at Huazhong University of Science and Technology (Wuhan, China), majoring in Environmental Engineering. In 2015, he started his master study in Environmental Sciences at Wageningen University & Research (Wageningen, The Netherlands). He carried out his master thesis research in the sub-department of Environmental Technology in which he studied anaerobic granular sludge formation under saline conditions. As a follow-up, he studied the dynamics of the microbial community during the granular sludge formation under saline conditions in the Laboratory of Microbiology. After he obtained his master degree, he sought opportunities to continue as a PhD candidate in the field of biotechnology and microbiology. In 2018, he started his PhD research related to converting wastewater treatment plants into polyhydroxyalkanoate production factories with a focus on recovering the polymer. The PhD research was a collaboration between Wetsus and TU Delft under the supervision of Alan Werker, Robbert Kleerebezem and Mark van Loosdrecht. In July 2022, he continued his journey in academia and started as a postdoctoral researcher at Wetsus, focusing on microbiology and microscope.

List of Publications

Published

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

5. **Pei, R.**[1], Estévez-Alonso, Á.[1], Ortiz-Seco, L, van Loosdrecht, M.C.M, Kleerebezem, R., Werker, A. (2022). Exploring the limits of polyhydroxyalkanoate production by municipal activated sludge. *Environmental Science & Technology*.

4. **Pei, R.**, Vicente-Venegas, G., van Loosdrecht, M.C.M, Kleerebezem, R., Werker, A. (2022). Quantification of polyhydroxyalkanoate accumulated in waste activated sludge. *Water Research*.

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

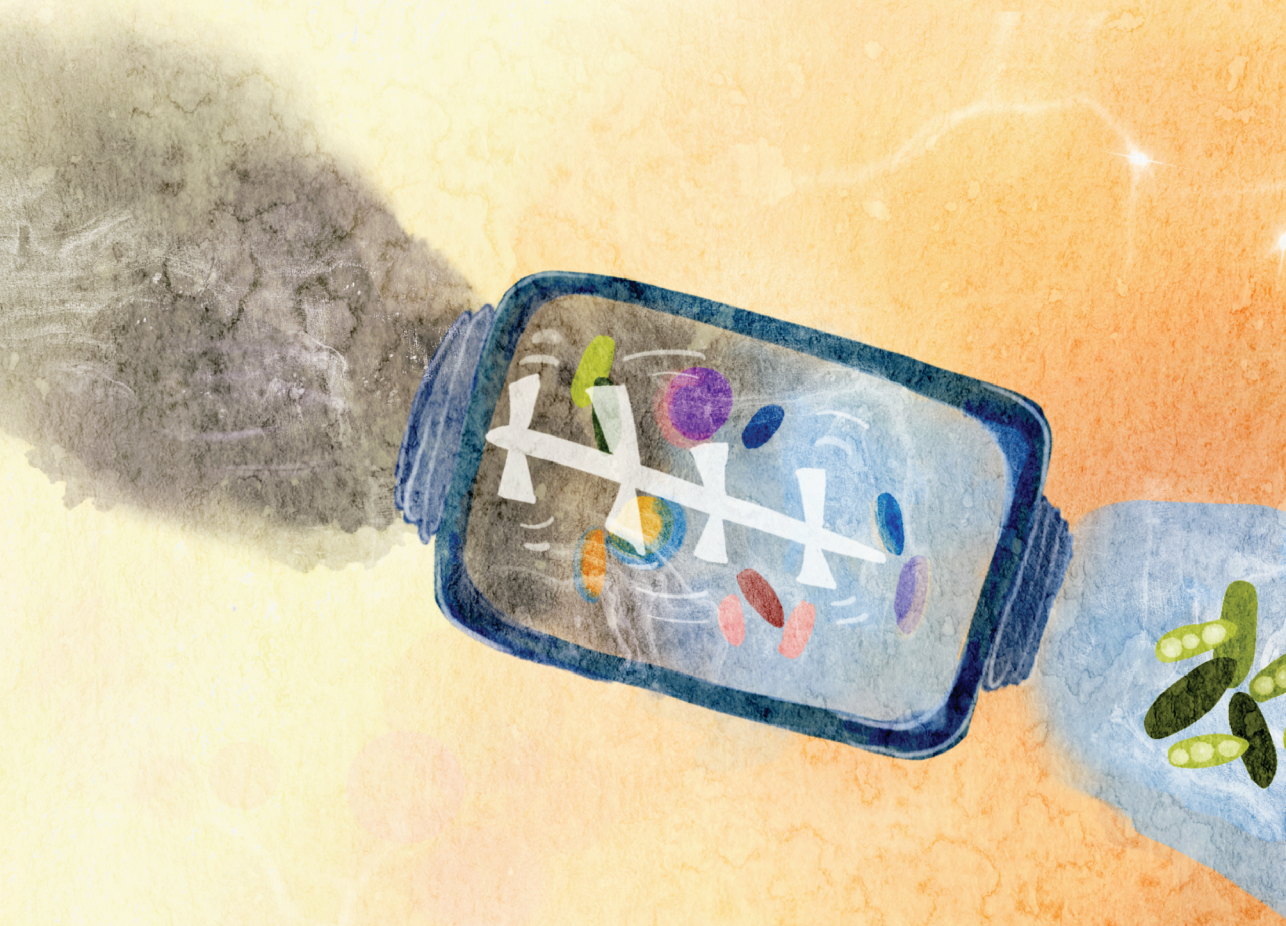
2. Gagliano, M.C., Sudmalis, D., **Pei, R.**, Temmink, H., Plugge, C. M. (2020). Microbial community drivers in anaerobic granulation at high salinity. *Frontiers in microbiology*.

1. Sudmalis, D., Gagliano, M.C., **Pei, R.**, Grolle, K., Plugge, C.M., Rijnaarts, H.H.M., Zeeman, G. and Temmink, H. (2017). Fast anaerobic sludge granulation at elevated salinity. *Water Research*.

Submitted

2. **Pei, R.**, Bahgat, N., van Loosdrecht,M.C.M, Kleerebezem, R., Werker, A.. Influence of Process Conditions on Accumulated Polyhydroxybutyrate in Municipal Activated Sludge.

1. **Pei, R.**, Vicente-Venegas, G., Tomaszewska, A., van Loosdrecht, M.C.M, Kleerebezem, R., Werker, A.. Visualization of polyhydroxyalkanoate accumulated in waste activated sludge.



Every microbe is a universe.