

# Consistent production of high quality PHA using activated sludge harvested from full scale municipal wastewater treatment – PHARIO

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## ABSTRACT

Production of polyhydroxyalkanoate (PHA) biopolymers by mixed microbial cultures concurrent to wastewater treatment is a valorization route for residual organic material. This development has been at pilot scale since 2011 using industrial and municipal organic residuals. Previous experience was the basis for a PHA production demonstration project: PHARIO. PHARIO was centred on processing surplus activated sludge biomass from the Bath full-scale municipal wastewater treatment plant in the Netherlands to produce PHA. Full-scale surplus activated sludge was fed to a pilot facility to produce PHA rich biomass using fermented volatile fatty acid (VFA) rich liquors from industry or primary sludge sources. A PHA rich biomass with on average 0.41 gPHA/gVSS was obtained with reproducible thermal properties and high thermal stability. A routine kilogram scale production was established over 10 months and the polymer material properties and market potential were evaluated. Surplus full-scale activated sludge, over four seasons of operations, was a reliable raw material to consistently and predictably produce commercial quality grades of PHA. Polymer type and properties were systematic functions of the mean co-polymer content. The mean co-polymer content was predictably determined by the fermented feedstock composition. PHARIO polymers were estimated to have a significantly lower environmental impact compared to currently available (bio)plastics.

**Key words** | activated sludge, bioplastic, mixed microbial culture (MMC), municipal wastewater treatment, pilot-scale, polyhydroxyalkanoates (PHAs), sewage sludge, volatile fatty acids (VFAs)

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## INTRODUCTION

Production of polyhydroxyalkanoate (PHA) biopolymers by mixed microbial cultures in combination with wastewater treatment is a potential valorization route for residual organic material and, previously, pilot plants demonstrated the technical feasibility of this route (Morgan-Sagastume *et al.* 2015; Bengtsson *et al.* 2017a). However, more information was needed about the reproducibility in quality of the PHA that can be produced in this way.

This has led to a PHA production and biobased value chain demonstration project: PHARIO (Bengtsson *et al.* 2017b). PHARIO was centred on processing surplus biomass from the Bath full-scale municipal wastewater treatment plant (WWTP) in the Netherlands to produce PHA polymers. The main objectives of the PHARIO project were to:

- prove that reproducible PHA product quality can be maintained using biomass harvested from a full-scale municipal WWTP;
- provide enough PHA material to be able to evaluate product quality and identify potential for market applications;
- understand the economic and environmental impact of this concept.

In this way, the PHARIO project is a stepping-stone to motivate further upscaling of the technology to a phase where the technology can be demonstrated as a production facility that provides enough material to establish downstream commercial applications. This manuscript is a summary of the extensive results of the PHARIO project. Information not shown or detailed herein may be found in the public STOWA report (Bengtsson *et al.* 2017b).

### PHA accumulation potential of municipal activated sludge

The possibility to produce a biomass capable of PHA accumulation with synthetic streams and process waters having relatively high concentrations of readily biodegradable chemical oxygen demand (RBCOD) has been studied for a long time (Valentino *et al.* 2017). More recently, it was found that the relatively low concentrations of RBCOD generally found in municipal wastewater are sufficient to produce biomass with a high PHA accumulation potential (PAP) given that a selective pressure for storage of RBCOD is established in the biological treatment process. It has been estimated that the PAP should be at least 40%

g-PHA/g-VSS for economical down-stream recovery of the polymer.

Before the PHARIO project, enrichment of a biomass with high PAP in a municipal WWTP had been observed at laboratory, pilot and, in one case, at full-scale treatment, namely at the WWTP Bath. However, until now it had been unclear how common enrichment of PAP is at existing wastewater treatment plants and what factors could influence the PAP level. Therefore, the PHARIO project aimed to clarify the wider scope for sourcing municipal activated sludge for PHA production by investigating fifteen WWTPs across the Netherlands with respect to PAP and the related process operating conditions. Activated sludge biomass grab samples were obtained from all the plants and assessed for PAP under standardized conditions in Sweden.

The outcomes (Bengtsson *et al.* 2017b) show clearly that high PAP in biomass at existing WWTPs is not a feature that is unique for one specific plant but is relatively wide spread even if a selection pressure for PHA accumulation potential has not been purposefully optimized in any of the locations. Among the fifteen plants tested in the PHARIO project, four plants (27%) had a PAP level of at least 38% g-PHA/g-VSS. With a PAP of 52% g-PHA/g-VSS, RWZI Beverwijk was well above a commercial threshold criterion of 40% and is therefore a very interesting resource for PHA production. A well-tuned municipal WWTP for PAP is considered to be able to achieve up to 60% PAP, given that specific attention is placed to ensure an optimal selection pressure for PHA accumulation. Based on the outcomes and the observations made on the sites, it was interpreted that the initial contact zone for biomass and wastewater RBCOD is a critical factor for driving PAP enrichment. Several alternative and relatively simple strategies are available for process modifications to impose and improve the quality of feast stimulation for the biomass. Optimization for PAP is likely to be possible with only minor process modifications.

## METHODS

### Pilot plant set-up

Production of PHA in PHARIO involved all four process elements that are required for this concept. It generally includes three biological process elements

(PEs): PE1 – acidogenic fermentation, PE2 – enrichment and production of biomass with PHA-storing capacity, and PE3 – PHA accumulation using PE2 surplus biomass and feedstocks with easily degradable organics. Polymers are recovered from a PHA-rich mixed culture biomass in a fourth process element (PE4) by means of solvent extraction (Arcos-Hernández *et al.* 2015).

PE1 was a pilot scale (1,200 L) well-stirred and thermostated batch anaerobic fermentation vessel called BioVAP that was operated on site at rioolwaterzuiveringsinstallatie (RWZI) Bath. BioVAP had been previously employed in a collaborative project to produce PHA with volatile fatty acids (VFAs) supplied by fermented Dutch agricultural residuals (Karlsson *et al.* 2014; Bengtsson *et al.* 2017a). PE2 was the full-scale WWTP process for active biomass production (RWZI Bath). PE3 was a pilot-scale (500 L) fed-batch process for PHA-rich biomass production using surplus PE2 activated sludge, and fermentation filtrate from PE1 as feedstocks (Morgan-Sagastume *et al.* 2015; Bengtsson *et al.* 2017a). PE3 was also operated on-site at RWZI Bath. PE4 was a pilot scale (10-L) batch green-solvent PHA recovery process (Werker *et al.* 2015).

In the normal routine of production operations, two accumulation batches were performed each week from June 2015 to March 2016. For each batch, a grab sample of freshly thickened (50 to 70 gTS/kg) surplus RWZI Bath activated sludge was obtained. The grab sample was assessed for solids content and about 1 kg of thickened activated sludge (as dry volatile solids) was delivered to the accumulation process along with dilution water. The accumulation pilot process comprised a working aeration volume of about 400 L coupled to a clarifier volume of 120 L (Morgan-Sagastume *et al.* 2015). The aerated volume was mechanically stirred, and air was supplied via a blower with coarse bubble aeration. Active pumping to, and return from, the clarifier maintained a short solids residence time in the clarifier. Depletion of dissolved oxygen for the biomass time in the clarifier maintained oxygen limiting kinetics which are known to prolong the metabolic activity associated with accumulation processes (Pratt *et al.* 2012). Therefore, time for the fractions of biomass in the clarifier at any one time was interpreted to be an accumulation dead time.

### VFA feedstocks

PHA was accumulated in the biomass by semi-continuous supply of a VFA rich feedstock. Organic sources for VFA feedstocks used in the project were either a fermented carbohydrate rich process effluent delivered from a local

candy factory, fermented primary sludge centrate from primary municipal sludge delivered from Waterschap De Dommel (Tilburg facility), and defined mixtures of acetic and propionic acids.

The process water from the candy factory was delivered batch wise once per week to RWZI Bath in intermediate bulk containers (IBC). The BioVAP pilot fermenter was used to maximize the fermentation product content in a well-stirred and temperature-controlled volume of 1,200 L. The process water was trimmed with dilution water to about 18 gCOD/L and was incubated at 37 °C for up to 7 days with a pH controlled nominally between 5.5 and 6.0. A 7-day incubation was not required for the fermentation, but this was time given conservatively and for simplicity with respect to all other aspects of the hands-on piloting production activities. The pH was maintained with a proportional–integral–derivative (PID) controller and pulse wise additions of concentrated NaOH (45% w/w). The fermentation process biomass (nominally about 1.2 gTSS/L) was maintained within the process at an SRT of between 7 and 10 days. Suspended solids from the candy factory process water fermentation effluent were removed by means of pre-settling with added flocculent polymer (FloPam FO 4800 SH, SNF Floerger), followed by a Hydrotech drum filter model 801 (HDF 801, with 10 µm screen). Suspended solids levels in and out of the HDF were on average 0.6 and 0.2 g/L, respectively. The fermentation product water for the accumulation process contained on average 16 gCOD/L as soluble RBCOD (readily biodegradable COD). Decrease in COD across the fermentation process was primarily due to water entrainment from the pilot HDF backwash operations. The feedstock nutrient balance of COD:N:P was 100:0.5:0.1 (by weight).

Primary sludge was also delivered fresh to RWZI Bath from the De Dommel Tilburg facility in IBC containers. Solids content was variable, but on average it was 45 g/L. The batches of delivered primary sludge were fermented with continuous mechanical stirring for 6 days at 37 °C, with pH monitoring but with no pH control. The sludge matrix was self-buffering and generally the pH was inherently maintained between 4.8 and 5.5. No solids were retained in the process between batches because unlike the process water from the candy factory, such sludge is already richly inoculated for anaerobic fermentation microbial activity. The fermenter batch was first discharged to a holding tank and then under automation control pumped to a centrifuge decanter (Morgan-Sagastume *et al.* 2015) with rotating bowl (3,000 rpm) and a relative velocity between bowl and screw of 10 rpm. Cationic polymer (FloPam FO4800SH, SNF/FLOERGER) was added in-line

for coagulation/flocculation to improve the solids/liquid separation. The polymer addition was set and controlled by programmable logic controller (PLC) to 50 g-polymer/kg-TSS. The solids reject cake was collected and disposed to the full-scale anaerobic digestion facility on site. Centrate was collected in a holding tank and iron chloride was added from concentrated solution (44% w/w) to precipitate excess soluble phosphorus based on a molar dosing ratio of 1.42 mol Fe:mol PO<sub>4</sub>. The centrate was further processed through the pilot Hydrotech drum filter (HDF 801, with 18 µm screen) to produce feedstock batches for the pilot-scale accumulation. This fermented primary sludge feedstock was about 7 gCOD/L with a COD:N:P balance of 100:5:0.1 (by weight).

So-called 'synthetic' feedstocks were generated by diluting and blending proportions of concentrated acetic and propionic acids in IBC containers to about 10 gCOD/L. A targeted COD:N:P (by weight) of 100:1:0.05 nutrient balance was obtained by chemical additions of NH<sub>4</sub>Cl and KH<sub>2</sub>PO<sub>4</sub>. pH was adjusted to 5 by the addition of NaOH (33 or 45% w/w).

### PHA accumulation

Prior to each accumulation, the biomass was subjected to an acclimation phase. This acclimation comprised a sequence of three feast and famine cycles. The feast in each cycle was generated by stimulating the biomass to near maximal respiration with a pulse input of substrate to a targeted maximum COD concentration of 200 mgCOD/L. The feast respiration response duration was measured based on changes in dissolved oxygen concentration. A period of famine was provided to last four times as long as the time of feast. The acclimation phase was designed as a controlled assessment of the biomass respiration response from batch to batch over the 10 months of operations, and as a means to provide all the biomass batches with the same perturbation history before the onset of the accumulation response. Previous work suggests that feast-famine acclimation before an accumulation response generally results in an improved PHA accumulation potential (Werker *et al.* 2016; Morgan-Sagastume *et al.* 2017).

After the last acclimation famine period, the accumulation process was started automatically. Accumulations were at 25 °C and were sustained using semi-continuous feedstock supply based on feed-on-demand biomass respiration control methods (Werker *et al.* 2013a). The control method objectives were designed to sustain a prolonged period of near maximal and continuous feast respiration

with pulse wise inputs of substrate. There is no requirement that the temperature should be 25 °C and equally successful results have been demonstrated for mixed culture accumulations over a wide temperature range from 15 to 30 °C (De Grazia *et al.* 2017). The accumulation or 'PHA production process' was typically maintained from 16 to 20 h in duration.

Several bioprocess optimization and performance efficiency improvements were not undertaken during the present PHARIO investigation because the main goal was to produce polymer as routinely, and as often, as possible. All efforts were on producing materials and evaluating the material quality with respect to the routine of production that was applied.

With exceptions of a few abnormal events that were detected by the process automatic control, the accumulation process was terminated based on a set time of accumulation. Due to the scheduling and logistics of production, as well as the permitted timing for access to the site the automated termination occurred frequently in the middle of the night. After accumulation termination, the aeration was turned off and the biomass was collected in the main process volume and allowed to settle by gravity. With return of operating personnel in the morning, and now under manual control, about 100 to 150 L of mixed liquor containing gravity thickened solids of PHA-rich biomass were pumped over to a 200 L holding tank and the mixed liquor pH was adjusted to 2 with concentrated H<sub>2</sub>SO<sub>4</sub> (Werker *et al.* 2013b). Following acidification, the solids were further thickened by flotation within the 200 L volume and these thickened solids were then dewatered by means of a filter bag centrifuge after adding sludge dewatering chemicals. The mixed liquor with added dewatering chemicals (FloPam EM 840 TBD) with a bag centrifuge (at 980 × g with filter bags defined with 7 L/dm<sup>2</sup>/min at 200 Pa) were dewatered on average to about 19% dry solids. Under optimized dewatering conditions, it is anticipated to reach approximately 25% dry solids content. The sludge cake was transferred manually to drying trays and dried at 70 °C.

### Production batches

In total, 59 activated sludge batches Axx (A01 to A59) were produced, with three different types of feedstocks: Sxx, Cxx and Pxx over 10 months of pilot operations. For Sxx, the xx denotes the propionic weight fraction (as COD) in the mixture of acetic and propionic acids. Thus, S05 denotes 5% propionic acid and 95% acetic acid as COD in the feedstock. Cxx refers to the fermentation batches with the process

water from the candy factory (C01 to C26) where C01 to C06 were made during the initial commissioning and benchmarking period. Pxx refers similarly to the primary sludge fermentation centrate batches P01 to P12 that were produced in the final and third phase of operations.

Fifty-two batches of PHA-rich biomass were successful from the 59 accumulation runs. The quality of the polymer in the biomass (PHA-in biomass quality), and the recovered polymer quality in relation to the feedstock and the operations form the basis of the assessments performed. Out of the 52 successful accumulation trials: 20 were with defined mixtures of acetic and propionic acids (S feedstock), 27 were with supply of fermented candy factory process water filtrate (C feedstock), and 5 were with supply of fermented primary sludge centrate (P feedstock).

### PHA extraction

PHA rich biomass was processed for reference (10 mL and 1 L) and pilot scale (10 L) polymer recovery. From all of the successfully 52-completed accumulation (Axx) batches, reference polymer recovery was made for all batches at the test tube scale with 10 g/L polymer loading, in acetone and at 125 °C for 1 h (Chan *et al.* 2017). In addition, 23 × 10 L PHA extractions were undertaken from selected Axx batches to supply a range of copolymer material types for investigations related to the quality and opportunities for commercial developments.

The pilot scale recoveries were undertaken as previously disclosed (Werker *et al.* 2015). Recoveries at the 10 L scale represented a range of types of PHBV blends with 3HV contents ranging from 0 to 44 weight percent. Recovered materials were further used in case study evaluations of routes to market by direct interaction with well-established commercial end users and product developers (compounders and processors). Pilot solvent extraction was with 2-Butanol, but other so-called PHA-poor solvents are possible (Werker *et al.* 2015). Recovered polymers were rinsed following recovery and pressing to more adequately remove residual solvent and to compensate for the non-industrial nature of the piloting equipment. Extraction and loadings were performed with the same protocol for comparison of the recovery reproducibility. Tuning of the recovery parameters for optimal conditions with respect to the different PHBV types was beyond the project scope, notwithstanding that this is a central and important principle for industrial process (Werker *et al.* 2015; Chan *et al.* 2017). Optimal conditions to recover PHB are not the same as those for a co-polymer blend of PHBV, nor a homopolymer

of PHV. The focus was on production and evaluation of the product value. A constant recovery solvent loading of nominally 50 g/L polymer was applied in all cases and, therefore, there was some variation in solvent biomass loading due to the minor variation in the batch to batch biomass PHA content. A 52-min heating program from 50 °C to a maximum solvent temperature of 140 °C was used and the average solvent temperature during the extraction heating cycle was nominally 103 °C. On average about 87% of the extractable polymer was targeted based on extraction rate evaluations (Werker *et al.* 2015).

Mechanical testing of the pure recovered PHARIO copolymers was undertaken in order to assess for the consistency of the material behaviour as a function of polymer type. PHARIO PHBVs were recovered at pilot scale from the accumulations representing a range of polymer types with respect to the mean 3HV content of the PHBV co-polymer blends.

## RESULTS AND DISCUSSION

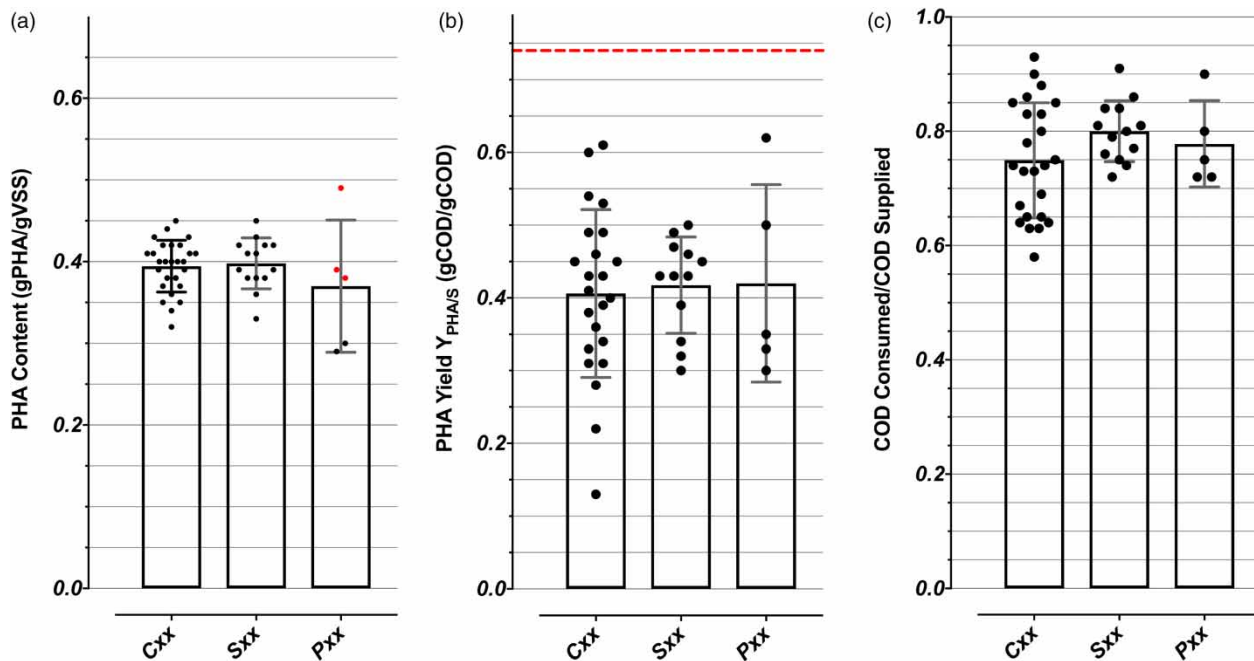
### PHA yields and substrate consumption

A dried 'PHA-rich-biomass' or PRB was routinely produced. Biomass is hygroscopic and normally in storage at room temperature the dried materials will contain about 2 to 4% bound water. Neglecting this moisture content the PRB from 52 accumulation batches comprised three fractions: non-PHA biomass ( $\approx 53$  wt%), inorganic material ( $10 \pm 2$  wt%) and PHA ( $37 \pm 5$  wt%).

We consider the PHA accumulation potential (PAP) with respect to the biomass organic content as this most closely reflects what the 'active biomass' is able to routinely achieve. This organic content is measured as volatile suspended solids or VSS. Therefore, on average, RWZI Bath activated sludge produced PHA with 41% gPHA/gVSS. Thus, we found that 1 kg of Bath activated sludge VSS, as the process is operated today, can be expected to reliably accumulate about 700 grams of polymer. As discussed above, there are ways to further improve the PHA accumulation potential for a municipal WWT activated sludge with expectation of up to 60% gPHA/gVSS, but this was not within the scope of the pilot operation.

The amount of polymer accumulated was with some variation but generally the product PHA content was consistent and not significantly influenced by the type of feedstock (Figure 1(a)). Variation in the case of the Pxx accumulation batches was due to the need for accumulation





**Figure 1** | (a) The PHA accumulation potential (PAP) was reproduced with three distinct types of feedstocks and associated matrices with and without nutrients in limitation. (b) Average accumulation yield of PHA produced per mass of feedstock substrate consumed. (c) Efficiency of the use of the COD in the feed.

process optimization when feeding a nutrient rich feedstock. We found that a higher specific loading of the Pxx substrate to the biomass (red symbols) gave PAP outcomes that were similar or better to the expectations based on Cxx and Sxx batches. It was further interpreted from the data for observed content variation with the Sxx accumulations where feedstock quality was constant, that managing biomass physiological state before the accumulation process was likely a factor to optimize in the future. By physiological state, we are referring to the extant metabolic state of the bacteria in advance of the accumulation process.

The average accumulation yield of PHA produced per mass of feedstock substrate consumed is shown in Figure 1(b). The efficiency of the biomass to accumulate PHA is best compared on the basis of grams of PHA produced (as COD) with respect to grams of substrate consumed (as COD). A COD basis removes the influence of the fact that different kinds of PHA contain different amounts of chemical energy. For example, PHB is 1.67 gCOD/g and PHV is 1.92 gCOD/g. A maximum theoretical efficiency of PHA storage is predicted to be about 0.74 gCOD/gCOD (Beun *et al.* 2002). This level of efficiency is generally only possible when the bacteria have little to no stored PHA. As the biomass becomes ‘fat’ with the intracellular granules, increasing maintenance energy requirements means that more of the feedstock carbon is spilled to carbon dioxide

rather than being converted and stored as polymer (Montano-Herrera *et al.* 2017; De Grazia *et al.* 2017).

Due to the trend of diminished polymer yield with accumulation time, an accumulation process should not be run longer than it needs to be run to reach maximal PHA content (De Grazia *et al.* 2017), unless concurrent growth of PHA storing biomass is to be stimulated (Valentino *et al.* 2015). The initial yields of PHA on substrate were generally high with reference to a recent review of the mixed culture PHA literature (Valentino *et al.* 2017). The literature values are with a wide range that depend on substrates but also on the basis of how the yields were calculated (initial versus after an arbitrary length of an accumulation process). Unfortunately, there has been no common standard in reporting such yields in the research literature referenced to, for example, the normalized accumulation time scale, and meaningful comparisons are therefore sometimes difficult to obtain. Reports of ‘high’ and ‘low’ yields in the literature can therefore be misleading if the context of the reported values is not clear. Without any context in the literature, average yield values between 0.4 and 0.6 gCOD/gCOD may be considered to be typical for mixed culture systems.

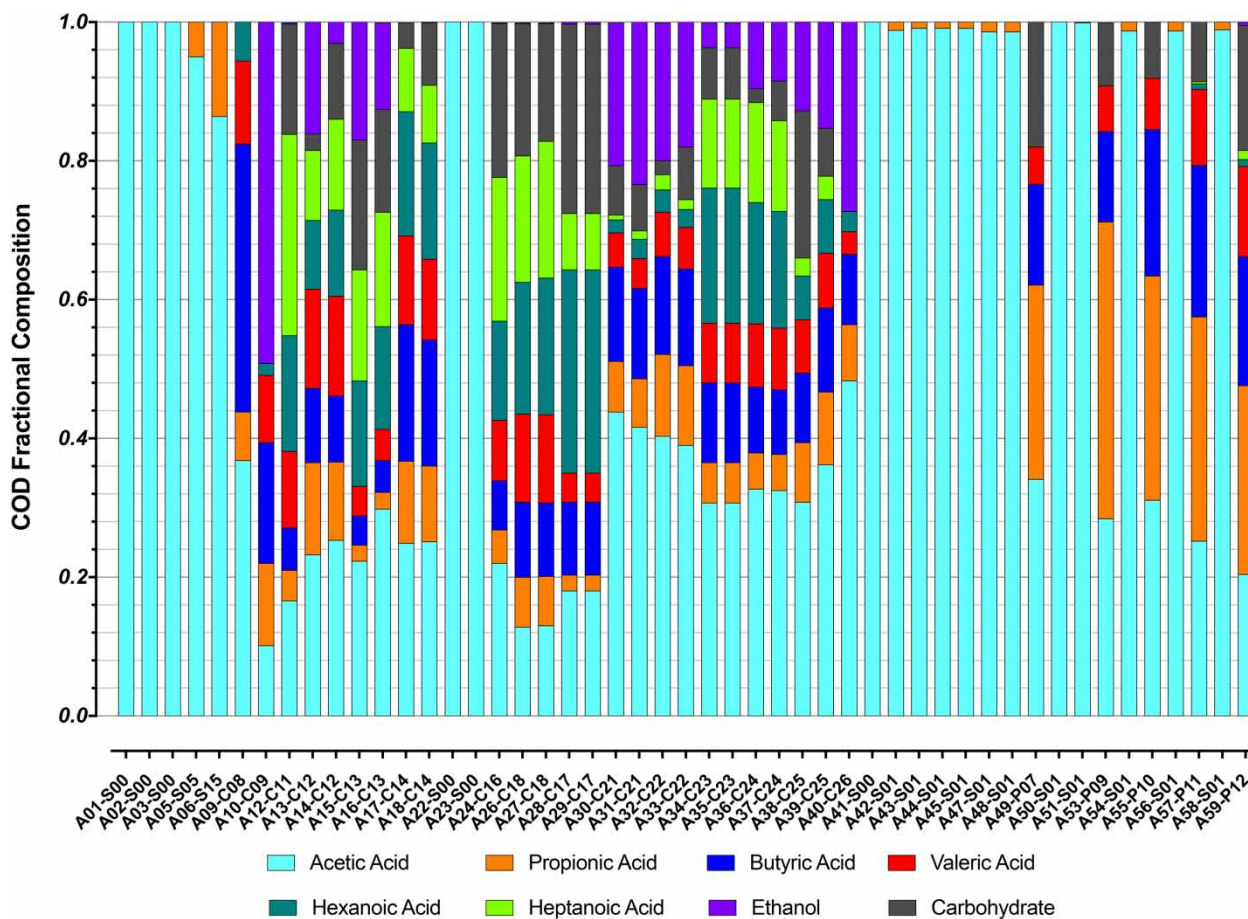
Alongside the potential for improvement and optimization of the biomass polymer yield on substrate, the overall pilot operations efficiency of substrate supply

was evaluated. The amount of substrate (as COD) consumed due to biological activity was estimated with respect to the amount of PHA produced for each Axx batch. The pilot system was operated with flow through, under hydraulic balance, meaning that every litre of feed added at the front end resulted directly in a litre of effluent discharged at the back end of the process. Bacterial biomass was retained in the pilot process by means of gravity separation. The estimated leakage (about 20%), of input feedstock COD short-circuited to the effluent, was not a surprise (Figure 1(c)). Methods of bioprocess operation and control to minimize feedstock leakage are known and these may be readily implemented. Such process control efficiency factors were beyond the scope of the pilot operations for PHARIO but are recommended for demonstration scale developments. Bacterial activity will also result in some production of soluble microbial products. This bacterial activity by-product COD that is not feedstock COD will nevertheless be interpreted as

non-consumed COD from the COD mass balance as it was estimated from these data.

### Feedstock production and composition

A range of feedstocks were used to produce PHBV co-polymer blends over the 10 months of pilot plant operations. The organic composition of the feedstocks used for respective Axx accumulation batches are shown in Figure 2. Sxx batches were synthetic feedstocks with defined mixtures of acetic acid with 0, 1, 5 and 15% COD fractions of propionic acid. Cxx batches were the BioVAP pilot filtrates from fermented process effluent from a local candy factory. Note that shifts in composition for the same Cxx feedstock (for example, C25 for A38 and A39) are due to continued fermentation that took place while the materials were in storage. Pxx batches were the BioVAP pilot centrate from fermented primary sludge delivered by Waterschap De Dommel from Tilburg.



**Figure 2** | Relative COD composition of the range of feedstocks used to produce batches (A01 to A59) of PHA-rich biomass over the 10 months of pilot plant operations. Non-VFA COD is expressed as carbohydrate. The feedstocks were batches of synthetic VFA mixtures (Sxx), fermented candy factory process effluent (Cxx), and fermented Waterschap De Dommel primary sludge centrate (Pxx).

The Pxx feedstock fermentation results were largely consistent and reproducible both at pilot and laboratory test tube fermentation scales. The low variability in the case of Pxx fermentate is related, in part, to the fact that hydrolysis is a rate limiting step to the fermentation biological activity. Furthermore, in primary sludge, the balance of carbohydrates, lipids and protein in sludge is expected to be relatively consistent. In contrast, the Cxx feedstock batch fermentation results were variable in nature. This variability was promoted from the onset by the current process effluent management practices at source. These stimulated a large and preventable level of yeast fermentation while the effluent was in storage at the candy factory. This alcohol fermentation activity could be readily prevented in the future. The history of the effluent storage and management at the candy factory today inadvertently encourages the maintenance of a significant yeast biomass. In addition to yeast alcohol production, the Cxx fermentation outcomes are also linked to challenges, in general, of fermenting a carbohydrate rich stream. These challenges are manageable but were beyond the scope of the study.

The BioVAP pilot fermentation process, as it was run for PHARIO, was not a suitable bioprocess method to constrain the possible degrees of freedom of fermentation metabolism. It was the fermentation infrastructure that was made available to the project before the sources of organic residuals were decided upon. The batch dump feed method of BioVAP meant that the carbohydrate conversion bioactivity was unregulated by the substrate supply as it should be. Conditions of pH and SRT could not be as readily controlled as they would be, for example, in an up flow continuous feed reactor (Zoetemeyer 1982). Notwithstanding, we found over the course of the project that other parameters (in addition to pH, SRT, and substrate limitation) could be used in the future to manipulate and steer the challenges due to alcohol production and secondary fermentation microbial activity. Nutrient regulation and the use of an electronic sink such as iron were both found to be effective control parameters for such refined and higher sugar content organic residual streams. Therefore, the observed variations in the Cxx feedstock fermentation products were interpreted to be controllable in scaled up fermentation for PHA production, through better source management practices and well-established methods of bioprocess operations and control.

The combination of a carbohydrate rich stream with a dump feed batch fermentation bioprocess, and the uncontrolled-at-source raw materials management for fermentation products were challenging in PHARIO, but

they were a benefit for PHARIO purposes. Cxx feedstock composition variation facilitated a benefit to the PHARIO objectives because it gave a deeper understanding of the reliable nature of polymer quality from an RWZI activated sludge biomass. We found that the activated sludge biomass from the full scale WWTP was a robust and stable raw material for polymer production over 10 months and four seasons of operations. The feedstock consistently determined the type of polymer produced, not the biomass.

### VFA feed composition determines co-polymer composition

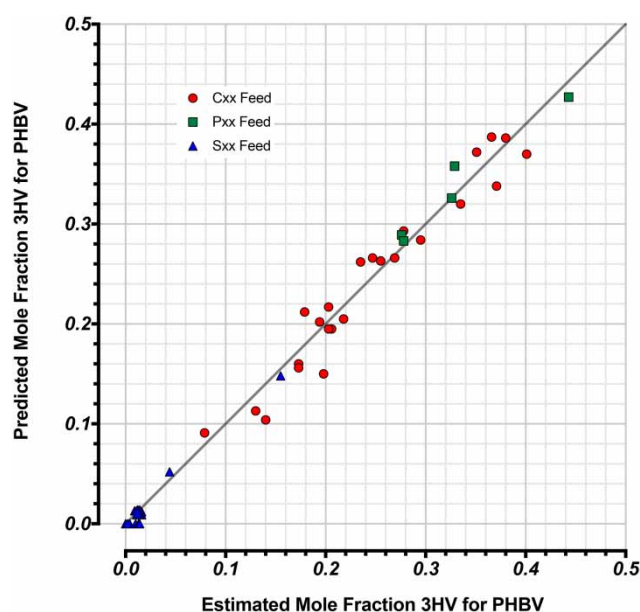
Due to the benefit of a broad range of feedstock compositions used in PHARIO, we produced a wide spectrum of co-polymer blends in the range from 0 to 50 wt.% 3HV in PHBV. It was of interest to understand if the feedstock composition was directly related to the average co-polymer blend composition. The conversions of the individual feedstock components into metabolic precursors for 3HB and 3HV are well described in the research literature. Metabolic models have been proposed to describe the pathways from substrates to polymers.

If the co-polymer composition was predicted by the feedstock composition, then that would suggest that the biomass is itself a robust raw material for producing the specific co-polymers of PHBV just by controlling the feedstock. The maximal molar contributions for 3HB and 3HV based on conversions of substrates to acetyl-CoA or propionyl-CoA were defined from the research literature. The fractional contributions of 3HB and 3HV derived from the respective substrates were estimated by minimizing the RMS error for the measured average 3HV polymer content.

Modelling results (Figure 3), after removal of justified outliers, supported the fact that, for the typical circumstances, biomass response of average accumulated biopolymer 3HV composition was directly related to the feedstock composition. These are results for a full scale activated sludge, sampled over 10 months of WWTP operations, over four seasons, and with three distinctly different feedstock sources. The standard deviation in the error for the predicted 3HV molar content of the PHBV was 1.6%.

There was no correlation of model errors to biomass PHA content, molecular weight, and yields of active biomass or PHA. However, higher ethanol feedstock concentrations resulted in poorer predictions of the PHBV 3HV content. Model predictions were further improved if the best fit coefficients were determined for Sxx, Cxx or Pxx feeds independently. This improved feedstock-specific





**Figure 3** | The measured PHARIO PHBV molar 3HV content from DSC was modelled with respect to the expected 3HV content based on the feedstock molar composition and constrained by known metabolic pathways.

model may indicate that other factors, and/or specific presence or absence of feedstock components like nutrients do exert some different influence on the biomass metabolism and conversion in producing PHBV. Pxx feedstocks were with relatively higher soluble nitrogen concentrations.

Drift in VFA composition for the same batch of fermented candy factory feedstocks occurred in some cases during storage between replicate accumulations with the same Cxx batch. In general, the type of PHBV co-polymer produced was a deterministic function of the feedstock quality and the biomass was a stable raw material from which PHA polymers can be produced. This outcome was a very exciting and important result for the proof of concept of the viability for mixed culture PHA production methods to yield predictable polymer products.

Average weight average molecular mass ( $M_w$ ) was more variable than expected from batch to batch, but nominally in the order of 500 kDa. The observed variability was interpreted to be dominated by a secondary downstream effect from the conservative choice to dry the biomass isothermally at 70 °C. The PHARIO project showed that a high initial drying temperature of at least 120 °C with well-defined cake morphology under drying is preferable. Future attention to detail for the biomass drying conditions are anticipated to yield much more consistent and higher downstream values (1,500 kDa) of the recovered polymer molecular weight.

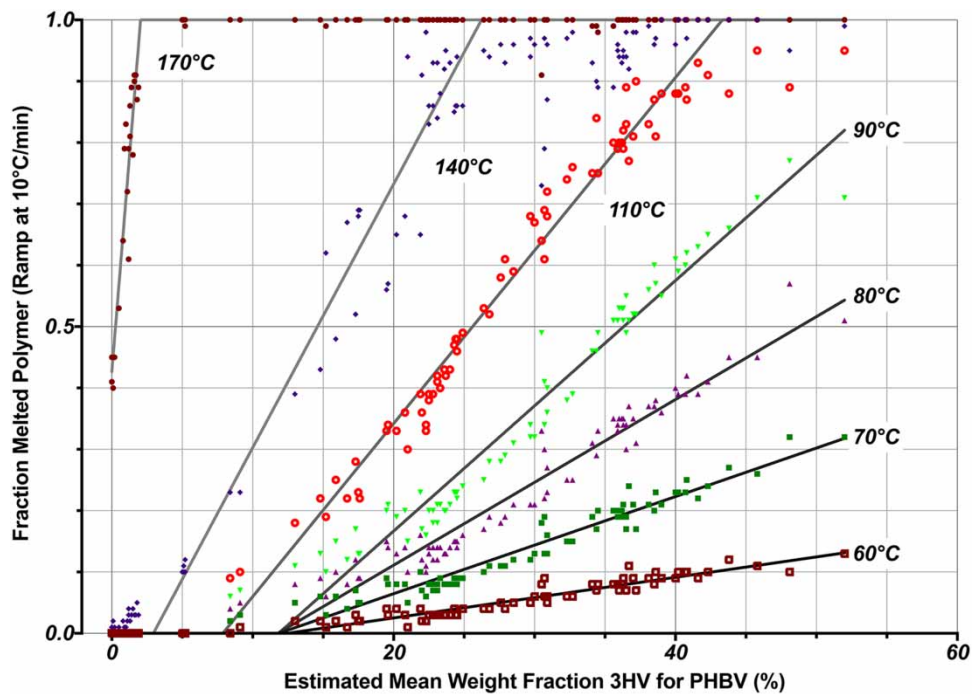
## Quality of the produced PHA

Product value will be very much linked to the reliability and predictability of the polymer melt characteristics. The co-polymer blends melt over a range of temperatures wherein the mean melt temperature decreases and the range increases as the co-polymer content goes from lower to eutectic average 3HV contents of about 50% HV. This outcome suggests that the higher 3HV co-polymer blends in this range contained a wider distribution of co-polymer types. A key factor for downstream utilization of the polymers is that the polymer properties behave systematically such that optimized formulations and processing conditions can be established towards any given value-added application for these materials.

Over a wide range of material type the *melt behaviour* of a given polymer type followed a definable trend (Figure 4) that was a simple function of mean 3HV content. The graph shows the fraction of polymer melted consistently as a function of temperature and average 3HV content.

The pilot-scale recovered polymers were melt processed into test elements for evaluating the material mechanical properties. Tensile test ‘dog bones’ were by hot press. Each dog bone was from a grab sample of 0.9 g of extracted purified PHBV powder disposed to a mould (thickness of 2 mm, width of 5 mm, and a gauge length of 15 mm). The mould was placed in the hot press at 180 °C and 30 bars for 5 min. After 5 min, the mould was cooled by water to 70 °C before removing the test specimen. Six test bars were made for each sample. Tensile testing was carried out according to ASTM D638 standard method using an Instron 430I tensile testing machine. A small pre-load (less than 5 N) was applied to the specimen at a cross-head speed of 0.1 mm/min and tensile testing was with a cross head speed of 10 mm/min.

Flexural test bars (1 mm × 6 mm × 40 mm) were similarly made by hot press. Flexural testing was carried out according to ASTM D790 standard method with the same Instron 430I tensile testing machine. The specimen was deflected until rupture on the outer surface or a maximum strain of 5.0% was reached, whichever came first. Note that it was a challenge to manufacture, on small scale and with limited materials identical test specimens without mould defects. Therefore, the mechanical test data results and trends were expected to exhibit a degree of variability. Notwithstanding this variability, the purpose of the testing was to evaluate the extent to which the mechanical properties were correlated with the polymer type and/or variation in the source material molecular weight. The absolute values



**Figure 4** | Fraction of polymer melted as a function of temperature and average 3HV content.

of the mechanical properties were of less interest than the display of systematic trends. With the benefit of the wide range of polymer types produced in PHARIO, it was possible to evaluate an influence of polymer type on the material mechanical properties. All specimens were aged for 2 weeks at room temperature between production and testing in order to allow for secondary crystallization.

Replicate specimens exhibited a degree of variation as expected in elasticity modulus and tensile strength (Figure 5). Properties of elasticity modulus and tensile strength correlated directly with the polymer type (i.e. average 3HV content). The weight average molecular mass ( $M_w$ ) varied between 200 and 600 kDa. However, errors between the line of best fit, in the correlation to 3HV content, did not suggest any influence of molecular weight on the mechanical properties in this range. This outcome is supported by other in-house observations and previous literature evaluations (Luo *et al.* 2002). Mechanical properties of these PHBV polymers in this  $M_w$  range are expected to be insensitive to  $M_w$  down to about 150 kDa. The scatter in the mechanical test results for the pure materials is believed to be mostly due to an influence of test specimen sample-to-sample variations and imperfections.

Within the framework of the PHARIO project more data were generated on the quality and control of the quality of the produced PHA product. For more information

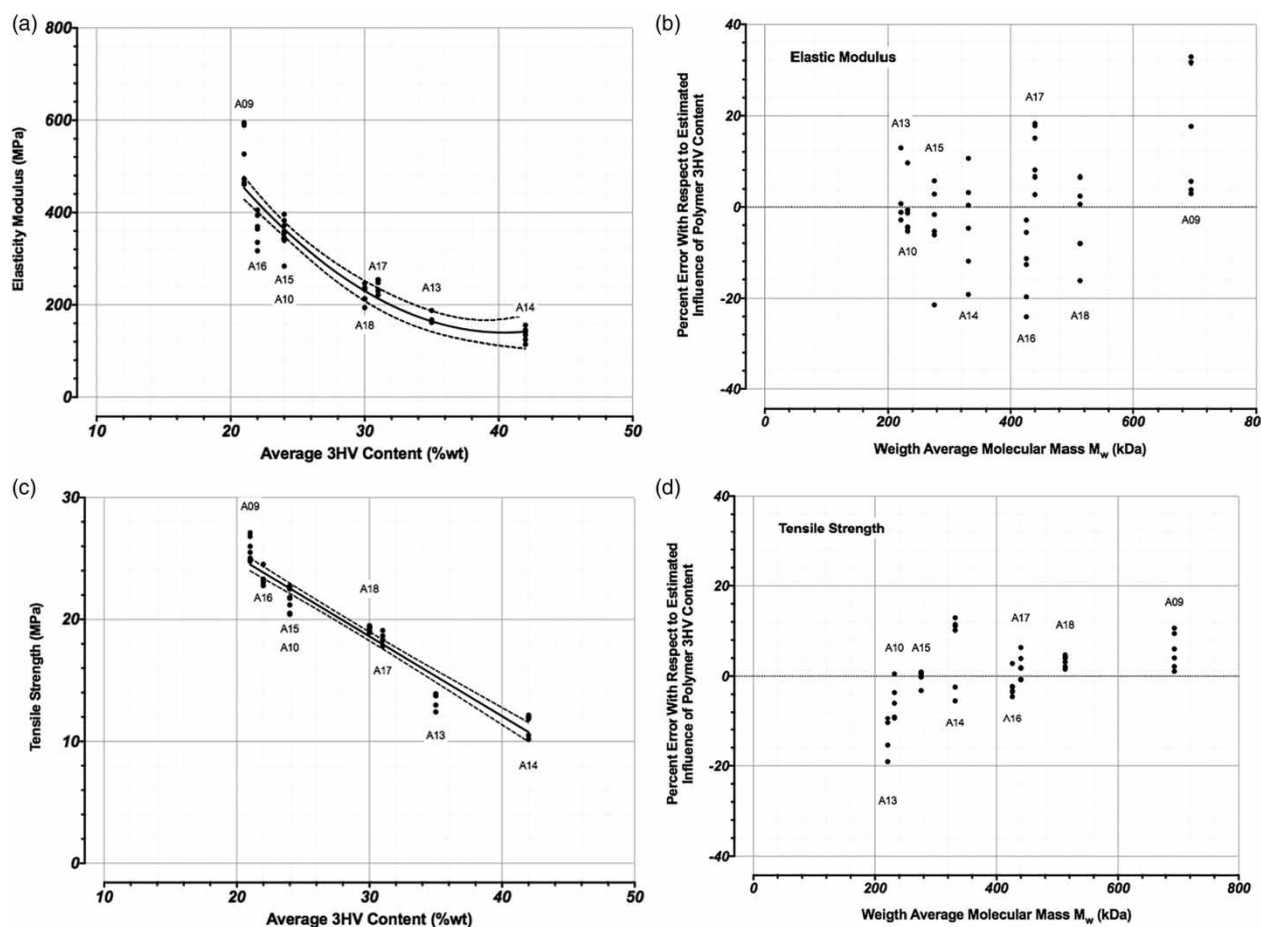
reference is made to the PHARIO public report (Bengtsson *et al.* 2017b).

### Application case studies

Following intensive characterization of the recovered PHA quality, several case studies were performed to understand the potential for value in application. For instance, the PHA was compounded according to proprietary commercial formulations to produce a bioplastic showing similarities to modulate mechanical properties as for the current bioplastics on the market today. Also, PLA was compounded with PHARIO PHBV as a toughness modifier to improve the PLA impact strength. These results are reported in (Bengtsson *et al.* 2017b) and gave confidence that PHAs sourced from mixed culture biomass can exhibit similar and even superior qualities to PHAs that are commercially sold today.

### Economic and life cycle evaluation

Within the framework of the PHARIO project, a life cycle analysis and an economic evaluation were performed and summarized here. The PHARIO public report (Bengtsson *et al.* 2017b) gives details of the analysis and approach that was followed.



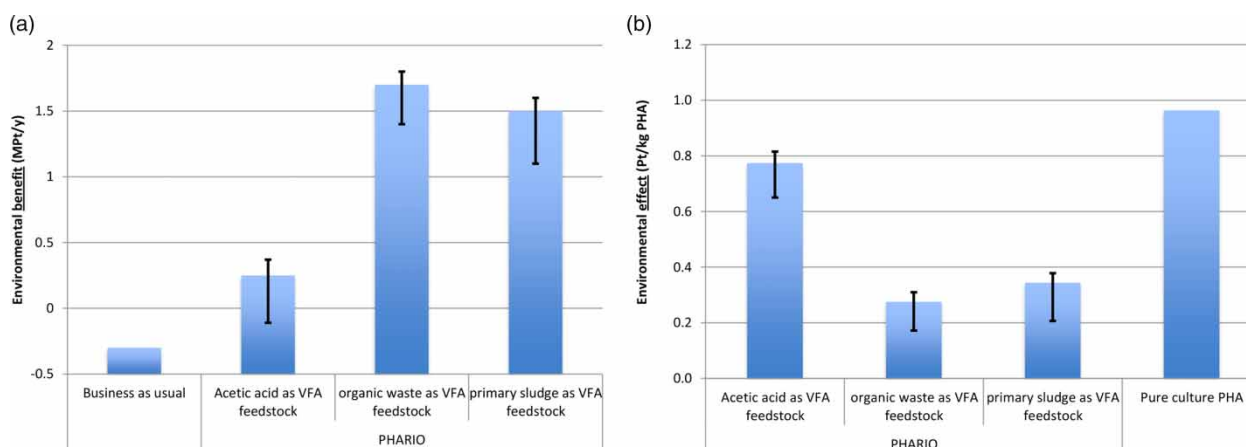
**Figure 5** | The mechanical properties of elasticity modulus and tensile strength were found to correlate with the polymer type (3HV content). The replicate test specimens exhibited a degree of variation as expected. Errors between the line of best fit and measurement values were evaluated and showed no well-defined correlation to  $M_w$  differences between samples.

Figure 6 summarizes the main outcome of the PHARIO LCA. The environmental effect can be evaluated in two different ways. First of all, the value chain with PHA production was compared to the situation where the sludge from Bath is treated in the conventional way (BAU). This comparison assumed there is an environmental benefit from the replacement of monoculture PHA by PHARIO PHA. All scenarios with PHA production lead to a significant increase in relative environmental benefit from  $-0.3$  MPt/year to  $+0.3$ - $1.7$  MPt/year, depending on the VFA source. Organic waste material as feedstock was estimated to bring the highest relative environmental benefit.

Producers of PHA bioplastics may prefer to know the environmental impact relative to PHA produced via monocultures that are available on the market today. Therefore, the environmental impact was calculated for production of 1 kg of PHARIO PHA. This approach showed that the PHARIO PHA was with a negative environmental footprint

of 0.3 Pt/kg PHA when organic waste or primary sludge is used as the VFA feedstock. Therefore, PHARIO PHA was estimated to have a 70% lower environmental footprint than PHA produced using monocultures. An environmental benefit of 20% even exists if pure commercial acetic acid would be used to produce the PHARIO PHA rather than waste residuals. The LCA outcomes were in agreement to a separate techno-economic assessment that was performed as part of the EU funding project ROUTES (Morgan-Sagastume *et al.* 2016).

Engineering designs for a full-scale commercial reference were made as part of PHARIO. The capacity of the PE3 accumulation facility was 2000 ton PHA/year (in PHA rich biomass). This capacity is typically fitting for a WWTP with a capacity of ca. 300,000 to 400,000 people equivalents. A central drying and extraction facility (PE4) was designed with production capacity of 5000 tons PHA/year. As a basis for the cost estimate, the following



**Figure 6** | Comparison of the environmental impact for the three different scenarios. The left figure compares the environmental impact of PHA production to the existing way of sludge treatment. The right figure compares the environmental footprint of PHARIO PHA to PHA produced via the monoculture route.

engineering documents were prepared: Process flow diagrams, equipment lists, site plans, and mass and energy balances.

The cost evaluation of a full-scale commercial reference plant estimated a manufacturing cost for the PHARIO of 3.5 €/kg PHA, including capital costs and based on the current state of the art. Current market prices were considered to be 4.5 €/kg or higher depending on PHA type and order size. The current costing was based on the pilot scale operations and already a number of potential cost reduction strategies have been identified that need to be verified at a larger scale. Therefore, the estimated production costs were considered to be reasonable with respect to the current level of development and accuracy of the estimations.

As a first next step, the concept should be scaled-up to provide enough material to test potential commercial applications. Without doubt, this phase will generate opportunities for further cost reduction and strengthening relationships with polymer application partners will further define the true market value of the produced material.

## CONCLUSIONS

1. The PHARIO project has found that full-scale municipal wastewater treatment plants can serve as process units that supply functional biomass (activated sludge) with PHA storing capacities even without modifications to the WWTP.
2. Within the scope of the PHARIO project, several wastewater treatment plants have been identified that have high PHA storing capacities. Other wastewater treatment

plants may require only minor modifications to achieve similarly high PHA storage capacities.

3. The PHARIO project demonstrated that a very high-quality product could be consistently made using municipal activated sludge as a raw material. Various opportunities for quality control were identified, giving confidence that products and quality control can be sustained despite the fact that the starting feedstocks are classified today as waste materials.
4. The LCA estimated that PHARIO PHA will have a 70% lower environmental footprint compared to current PHA plastics due to the use of the third-generation feedstock.
5. Estimations at basic engineering level showed competitive manufacturing costs compared to current market prices. Further cost reductions will be possible as a result of the project learning curve.
6. In the next stage, demonstration production of PHARIO PHA will facilitate developments to optimize the technology, and its economics, as well as generating the context from which business relationships models can be developed from practice rather than speculation.

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