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Pilot-Scale Polyhydroxyalkanoate Production from Organic Waste: Process Characteristics at High pH and High Ammonium Concentration

Michel Mulders[1]; Jelmer Tamis[2]; Ben Abbas[3]; João Sousa[4]; Henk Dijkman[5]; René Rozendal[6]; and Robbert Kleerebezem[7]

Abstract: Polyhydroxyalkanoate (PHA) accumulating microbial enrichment was established on volatile fatty acids (VFAs) containing leachate derived from the organic fraction of municipal solid waste (OFMSW). The enrichment was based on a 12-h feast-famine batch cycle and an exchange ratio of 50% in which VFAs were completely consumed in less than 50 min during stable periods of operation. No pH control was applied in the system, and the pH went as high as 9 due to the presence of amongst others, ammonia [500 mg · L^{-1} total ammonia nitrogen (TAN) on average]. The degree of enrichment was evaluated with fluorescence in situ hybridization (FISH), and a yet unknown genus of large (3–5 μm diameter) beta-proteobacteria appeared dominant in the culture. A method for estimating the fraction of PHA accumulating active biomass in the total volatile suspended solids was established, and the results indicated an increase of this fraction from 25% to 56% after implementing two modifications in the operational protocol: (1) a pretreatment of the substrate removing virtually all settleable solids; and (2) a settling phase in the enrichment reactor after the feast phase, selectively removing nonsettleable solids and slowly degradable substrates. The PHA accumulation potential of the culture was 77 ± 18 wt% PHA (n = 3) after 3 h in batch accumulation experiments. The results suggest the potential feasibility of PHA production under conditions that were previously considered economically favorable but technically difficult. **DOI: 10.1061/(ASCE)EE.1943-7870.0001719.** © *2020 American Society of Civil Engineers.*

Introduction

In the Netherlands, around 1.4 Mton of the organic fraction of municipal solid waste (OFMSW) was generated in 2017 (CBS 2018). Roughly ⅔ of this waste was directly being processed aerobically to compost and used as soil fertilizer. The remaining ⅓ of OFMSW was anaerobically digested to methane before being processed into compost. In the anaerobic digestion (AD) process, OFMSW is first hydrolyzed to yield monomeric compounds of proteins, carbohydrates, and lipids. Secondly, volatile fatty acids (VFAs) are produced from the monomeric compounds via fermentation. In the last two phases, the produced VFAs are further degraded via acetic acid, H_2, and CO_2 to methane containing biogas. An interesting alternative to compete with biogas production while treating OFMSW could be the production of PHA bioplastic (Kleerebezem and van Loosdrecht 2007).

The production of PHA from wastewater with open microbial communities has been widely studied (Colombo et al. 2017; Johnson et al. 2009; Korkakaki et al. 2016a), both in the lab and in various pilot studies (Bengtsson et al. 2008; Tamis et al. 2014, 2018; Valentino et al. 2018). A common strategy to enrich for PHA producing organisms is the application of a feast-famine regime. Lab studies have reported biomass with PHA contents of more than 85 wt% (gPHA · $gVSS^{-1}$ · 100%) on synthetic wastewater (Johnson et al. 2009; Marang et al. 2013). More variable and, in general, lower PHA contents (29–75 wt%) have been reported when using real wastewater in lab and pilot studies (Korkakaki et al. 2016a; Tamis et al. 2018). Commonly, these numbers are lower because of nonPHA solids in the wastewater and/or the presence of organic substrates not suitable for PHA production.

One interesting stream for the production of PHA is solid organic waste; but information about the suitability of this substrate is scarce until now, and it is worthwhile to investigate in more detail (Colombo et al. 2017; Valentino et al. 2018). The use of a liquid stream (leachate) derived from organic waste could pose difficulties for microbial PHA production due to its variation over seasons and its complex unknown structure. In this study, a leachate was investigated that was derived from organic waste for the production of methane containing biogas according to the Biocel process (Ten Brummeler 2000). Characteristics for this type of leachate are high NH_3 content, high alkalinity, and the presence of undefined soluble compounds and undefined particulate solids. Previous PHA production pilot studies were conducted on wastewater from a candy bar factory and papermill wastewater (Tamis et al. 2014, 2018). These wastewaters typically have a high chemical oxygen demand (COD) to nitrogen ratio and low amounts of undefined (particulate)

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Fig. 1. Impression of the pilot plant. The open container contained the selector and accumulator bioreactor (red bracket). In the front, there is an IBC sedimentation tank (orange bracket). Behind the sedimentation tank, the influent buffer vessel was located (yellow bracket). (Image by Michel Mulders.)

organic compounds. Furthermore, these pilot studies were operated in a reactor system with pH control and an operating pH of 7. In this study, the leachate had high alkalinity, rendering pH control expensive. The relatively harsh conditions encountered when using organic waste leachate were reflected in the results from a lab study conducted on similar leachate (Korkakaki et al. 2016a) that yielded a poor PHA accumulating potential (29 wt%). Only when the microbial enrichment was established on artificial VFA mixture, high PHA contents (78 wt%) could be achieved when fed with leachate during accumulation.

In this study, a PHA producing feast-famine enrichment without pH control was investigated on the pilot-scale, and an impression of the pilot is shown in Fig. 1. The substrate was leachate derived from the OFMSW collected on-site at Orgaworld Vergisting Biocel in Lelystad, Netherlands. In this work, we do not aim for the maximization of the PHA production per unit of OFMSW treated because the main fraction of VFAs was converted to methane containing biogas. The main research question of this study was whether a good overall PHA production process could be established with a complex feedstock like the leachate of OFMSW. The main criteria used to identify the process performance were the overall PHA production yield ($gPHA \cdot gCOD^{-1}$), PHA content of the product ($gPHA \cdot gVSS^{-1}$), and process stability.

Materials and Methods

A pilot plant situated at the Orgaworld Vergisting Biocel located in Lelystad, Netherlands, was operated for a period of 757 days. A period of 188 days was the focus of this study in which the pilot plant was operated under a modified version of the standard feast-famine operation, as described by Johnson et al. (2009). The modified version included a settling phase of 60 min after depletion of the rapidly degradable COD.

Substrate Production

The leachate used in this study was produced at the Orgaworld Vergisting Biocel located in Lelystad. At this site, the source-separated OFMSW was treated in anaerobic tunnel digesters. Fresh OFMSW was batch-wise mixed with digested OFMSW from the previous batch, which served as the seeding material. The OFMSW was anaerobically digested to methane containing biogas (Ten Brummeler 2000). Leachate from the tunnels was collected centrally and recirculated over the OFMSW tunnels continuously to enhance the transport of substrates between zones in the bed with more or less methanogenic activity. Leachate for feeding the PHA pilot was withdrawn from the central point where leachate was collected.

The collected leachate for the PHA pilot plant was first stored in a 1 m^3 intermediate bulk container (IBC). The leachate was stored for at least 24 h to allow settling of particulate material; after this period, the supernatant (ca. 500 L) was pumped in a buffer vessel of the pilot plant. Finally, the leachate was diluted 2–3 times toward a final concentration of soluble COD (COD_{Sol}) of 6 $gCOD_{Sol} \cdot L^{-1}$. The buffer vessel was 1,500 L and kept at 35°C ± 5°C, the substrate was not mixed, promoting further settling of solids, thus minimizing solids entering the bioreactors. Diluted leachate was in the buffer vessel for roughly 3 days, and after which, the buffer vessel was completely emptied and cleaned before being filled again with the new substrate, minimizing the methanogenic activity in the buffer vessel.

Enrichment Reactor

Enrichment of a PHA producing microbial community was conducted in an aerobic reactor. This reactor had a working volume of 180 L and was inoculated with a mixture of a lab enrichment, dominated by *P. acidivorans*, and activated sludge from a domestic wastewater treatment plant (Dokhaven, Rotterdam, Netherlands). Air was supplied via a fine bubble diffuser at a rate of $100\,L \cdot min^{-1}$ to prevent oxygen limitation and ensure mixing of the reactor broth. The reactor was kept at 30°C ± 3°C through a warm water jacket on the outside of the reactor. The pH of the system was only monitored and not controlled.

A selective environment that favors PHA production was created using a feast-famine regime. The sequential batch reactor (SBR) was operated using a cycle length of 12 h, solid retention time (SRT) of 24 h, and hydraulic retention time (HRT) of 17 h. A cycle consisted of the following phases:

1. *Feed phase (0–16 min)*: During the feed phase, 125 L of the substrate was supplied from the buffer vessel to the reactor for 16 min, resulting in a working volume of 177 L. In the leachate, the COD_{Sol}:P ratio was 275 ($gCOD_{Sol}$:gPO_4—P), and phosphorus was assumed to be limiting. Although all nutrients (except phosphate) were present in excess in the wastewater, additional growth nutrients were supplied in order to make sure that no nutrient limitation would occur. Nutrients were dosed to the reactor, 80–100 mL $cycle^{-1}$, throughout all phases, except for the settling phase. The nutrient mix consisted of the following components: 3 M urea-N, 0.3 M phosphate, 0.3 M $MgSO_4$, 0.2 M K_2SO_4, 64 mM $FeCl_3$, 3 mM $ZnSO_4$, 2.7 mM H_3BO_3, 2.1 mM $NiCl_2$, 1.5 mM $CoSO_4$, 0.6 mM $CuSO_4$, 0.8 mM Na_2MoO_4.
2. *Reaction phase (16–50 min)*: The reaction phase followed the feed phase. In this phase, the microorganisms had the opportunity to consume all readily biodegradable substrate aerobically.
3. *Settling phase (50–110 min)*: 50 min after the cycle initialization, a settling phase started, and during this phase, aeration was turned off for 60 min. After 60 min settling, the top half of the liquid (86 L) was removed from the middle of the reactor.
4. *Growth reaction phase (110–705 min)*: After effluenting the top half of the liquid, the aeration was turned back on for the remainder of the cycle. No substrate was added to the reactor.

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5. *Effluent phase (705–720 min)*: At the end of the cycle, around half of the remaining reactor broth was withdrawn (40 L). This biomass containing effluent was used optionally in the subsequent accumulation step.

Accumulation Reactor

A separate reactor was used to maximize the PHA content, and this reactor had a maximum working volume of 180 L. The reactor was inoculated with 10 L mixed broth from the enrichment reactor. Subsequently, the reactor was filled with 170 L of the substrate from the influent buffer tank. Aeration was similar to the enrichment reactor: $100\ \mathrm{L \cdot min^{-1}}$, and the temperature was kept at 30°C ± 2°C. After 4 h, the PHA-rich biomass was harvested using a pilot-scale centrifuge and processing a flow of $200\ \mathrm{L \cdot h^{-1}}$ at 3,000 g.

Sampling and Analytical Methods

Data were collected over a period of 188 days according to the sampling and measurement scheme shown in Table 1. Furthermore, to gain more insight into the process, multiple detailed sampling campaigns were executed. During these campaigns, the number of samples withdrawn from the reactor was increased (every 10 min during the feast period, every hour for 2–3 h in the famine period and accumulation reactor).

Samples withdrawn from the reactor for VFA, $\mathrm{COD_{Sol}}$ and TAN content were filtered before measurement (0.45 μm pore size, PVDF membrane, Millipore, Ireland). TAN and $\mathrm{COD_{Sol}}$ were measured using a commercially available spectrophotometric test cuvette kit provided by Hach-Lange. LCK302 was used to quantify the TAN, and LCK014 was used for $\mathrm{COD_{Sol}}$. The VFA content of the samples was measured using gas chromatography (GC). The GC was equipped with a ZB-WAXplus column (20 m length × 0.18 mm internal diameter, 0.18 μm film) and a flame ionization detector (FID), as described in Cabrera-Rodríguez et al. (2017), although in this study, iso-hexanoic acid was used as internal standard instead of anisole. The total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to standard methods (Clesceri et al. 1999). The SVI30 was determined as follows: The SV30 was obtained in an Imhoff cone according to standard methods (Clesceri et al. 1999) and then divided by the corresponding TSS of the sample minus the TSS of the influent. The PHA content of the dried matter was analyzed according to the method described by Johnson et al. (2009). The PHA content was extracted and esterified using a mixture of propanol: HCL (4:1) (1.5 mL) and dichloroethane as solvent (1.5 mL) for 3 h at 100°C. After separation of the solvent phase from the water phase, the PHA content (in the solvent phase) was quantified using a GC (model 6890N, Agilent) equipped with an FID on an HP Innowax column.

Data Analysis

The VFA fraction of the total soluble COD ($\mathrm{COD_{VFA}}$) concentration was determined as the sum of the individual COD concentrations of acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate. The amount of catalytic biomass was estimated by subtracting the amount of PHA and inert VSS from the total amount of VSS measured in the bioreactor. The VSS present in the pretreated leachate was assumed to be inert VSS. For practical purposes, it was assumed that $Y_{VFA}^{X_{PHA}}$ equals $Y_{BOD_{other}}^{X_{other}}$.

Therewith, the fraction of PHA producing biomass in the total catalytic biomass and the amount of catalytic biomass could be approximated as follows:

$$f_{XPHA} = \frac{\Delta COD_{VFA}}{\Delta COD_{sol}} \tag{1}$$

$$VSS_{catalytic} = (VSS_{EoC} - PHA_{EoC} - VSS_{inf}) \cdot V_{EoC} \tag{2}$$

$$BOD_{other} = \Delta COD_{sol} - \Delta COD_{VFA} \tag{3}$$

Table 1. Overview of the samples withdrawn, from each vessel/reactor and its frequency

Measurement	Sample point	Sample time	Frequency	Method
TSS/VSS[a]	Buffer vessel	SoC[b]	Daily	Wet-/Dry-/Ash-weight
	Enrichment reactor	EoC[c], EoF[d]		
SVI30[e]	Enrichment reactor	EoC, EoF	Daily	Imhoff cone
COD[f]	Buffer vessel	SoC	Daily	Spectrophotometric (Hach-Lange)
	Enrichment reactor	EoC, EoF		
Alcohol/VFA[g]	Buffer vessel	SoC	Daily	GC
	Enrichment reactor	EoC, EoF		
PHA[h]	Enrichment reactor	EoC, EoF	Daily	GC
NH_4^+	Buffer vessel	SoC	Every other day	Spectrophotometric (Hach-Lange)
	Enrichment reactor	EoC, EoF		
Conductivity	Buffer vessel	SoC	Daily	Handmeter
	Enrichment reactor	EoC, EoF		
pH	Buffer vessel	Continuous	Online	Ag/AgCl electrode
	Enrichment reactor	Continuous		
DO[i]	Enrichment reactor	Continuous	Online	LDO Sensor

[a]TSS/VSS stands for total suspended solids and volatile suspended solids.
[b]SoC stands for the start of the cycle.
[c]EoC stands for the end of the cycle.
[d]EoF stands for the end of the feast.
[e]SVI30 stands for sludge volume index 30.
[f]COD stands for chemical oxygen demand.
[g]VFA stands for volatile fatty acid.
[h]PHA stands for polyhydroxyalkanoates.
[i]DO stands for dissolved oxygen.

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The amount of PHA producers was estimated by using the fraction of COD being consumed by the PHA producing biomass (f_{xPHA}) multiplied with the approximated amount of catalytic biomass.

The expression calculating the amount of PHA producers X_{PHA} was formulated as follows. Furthermore, it was assumed no growth occurred using particulate COD

$$X_{PHA} = f_{XPHA} \cdot VSS_{catalytic} \tag{4}$$

The VFA specific substrate uptake rate q_{VFA} ($gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$) for the PHA producing biomass was estimated as follows:

$$q_{VFA} = \frac{\Delta COD_{VFA}}{X_{PHA} \cdot feastphase} \tag{5}$$

The VFA specific substrate uptake of all the VSS q^*_{VFA} ($gCOD_{VFA} \cdot gVSS^{-1} \cdot h^{-1}$) was estimated as follows:

$$q^*_{VFA} = \frac{\Delta COD_{VFA}}{VSS_{EoC} \cdot V_{EoC} \cdot feastphase} \tag{6}$$

The overall substrate uptake rate of all the VSSq^*_{sCOD} ($gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$) was estimated as follows:

$$q^*_{sCOD} = \frac{\Delta COD_{sol}}{VSS_{EoC} \cdot V_{EoC} \cdot feastphase} \tag{7}$$

For the accumulation experiments, the amount of VSS was measured at the beginning of the accumulation experiment to obtain the specific substrate uptake rates. Furthermore, instead of the length of the feast phase, the duration of the experiment was used.

The overall biomass production yield ($gVSS \cdot gCOD_{Sol}^{-1}$) was estimated as follows:

$$Y^{\frac{X}{sCOD}} = \frac{(VSS_{EoC} - PHA_{EoC} - VSS_{inf}) \cdot Volume_{EoC}}{\Delta COD_{sol}} \tag{8}$$

The overall PHA production yield was calculated using the method proposed in Bengtsson et al. (2008). First, the amount of PHA biomass (X^*_{PHA}) that could be produced from 1 $kgCOD_{Sol}$ was calculated. Next, the amount of PHA (PHA*) required to reach the target wt% PHA could be calculated using (X^*_{PHA}). Finally, the amount of PHA* was used to calculate the amount of COD_{Sol} required for its production, which was added to the 1 $kgCOD_{Sol}$ used for biomass production, resulting in an overall PHA production yield

$$X^*_{PHA} = 1\ kgCOD_{sol} \cdot Y^{\frac{X}{sCOD}} \tag{9}$$

$$PHA^* = \frac{X^*_{PHA}}{1 - PHA(wt\%)} - X^*_{PHA} \tag{10}$$

$$Y_{Overall\ PHA\ production} = \frac{PHA^*}{PHA^* \cdot \frac{1}{Y^{\frac{PHA}{\Delta COD_{VFA}}}} + 1\ kgCOD_{Sol}} \tag{11}$$

Microbial Community Analysis: gDNA Extraction, Cloning, and Sequencing of Full 16S-rRNA Genes

Full 16S-rRNA genes (~1500 bp), for the purpose of the development of specific FISH probes, were generated by performing a full gene amplification on a sample from the PHA enrichment reactor (May 2017). The first step was the extraction of the genomic DNA by using the UltraClean Microbial DNA Isolation Kit (Mobio) according to manufacturer's protocol, except a combination of heating (65°C) and beat beating was used (minibeater-16, Biospec). Following extraction, PCR amplification was done by using the primers GM3 and GM4 (Muyzer et al. 1995). The amplified fragments were checked and quantified using an agarose gel, purified using the QIAquick PCR purification kit (QIAGEN, Germany), and used for TOPO TA Cloning (Thermo Fisher Scientific). In total, 48 clones were picked from the plate and used for direct PCR on the plasmid using the primers MF, MR. The final product was checked on an agarose gel, and all clones yielded near 1600bp fragments. All these clones were sent for (bi-directional Sanger) sequencing to Baseclear, Leiden, Netherlands. The sequences were aligned and quality trimmed using CodonCode Aligner v.4.2.7. Software (Codoncode), resulting in complete 16S-rRNA genes with the primers sites removed. The final consensus sequences were exported and imported into ARB version 5.2 software (Ludwig et al. 2004), and redundant, identical, sequences were removed. The resulting 12 sequences, which were near identical (>99%), were used for probe development (implemented in the ARB software package). Several probes were developed with different properties in various positions. In the end, probe JT01 was most specific (matching only the imported sequences) after optimization. The unambiguous clone JT01 that represented all 12 clones was submitted to genbank (NCBI) under the accession number MK575517.

The microbial community composition was analyzed microscopically using FISH with a mixture of the probes JT01, UCB823, and EUB338I-III. A more detailed version of performing this FISH technique can be found in Johnson et al. (2009). Commercially synthesized probes with either 5' FLUOS or the sulfoindocyanine dyes Cy5 and Cy3 (Thermo Hybrid interactive, Ulm, Germany) were used, as summarized in Table 2.

Table 2. Oligonucleotides probes used for FISH analysis used in this study

Code	Sequence (5′-3′)	Specificity	References
EUB338 I	gctgcctcccgtaggagt	Bacteria	Amann et al. (1990)
EUB338 II	gcagccacccgtaggtgt	Bacteria	Daims et al. (1999)
EUB338 III	gctgccacccgtaggtgt	Bacteria	Daims et al. (1999)
UCB823	cctccccaccgtccagtt	*P. acidivorans*	Johnson et al. (2009)
JT01	tccacacacgctattcacgca	Clone JT01	This study

Results

Characterization of the Substrate

Pretreatment of the substrate aimed at minimizing suspended solids concentrations in the influent. To this end, the OFMSW-leachate used in this study was stored at ambient temperatures for at least 24 h in a 1 m^3 IBC vessel prior to usage. After storage, the upper fraction was stored in the substrate buffer vessel. The leachate had a COD_{Sol} concentration of $16.6 \pm 5.6\ gCOD_{Sol} \cdot L^{-1}$ (average ± standard deviation; n = 75), and this was diluted with tap water to 6 $gCOD_{Sol} \cdot L^{-1}$ before being used in the PHA production pilot. A summary of the substrate properties as measured in the influent buffer vessel (after dilution) is shown in Table 3.

Selector Characterization

Reactor operation was started in January 2016, and data collection started on March 29, 2016. The enrichment was monitored for a period of 667 days. Within 13 days after start-up of the reactor, typical feast-famine dynamics were observed (Fig. 3), and the length of the feast phase was identified using the dissolved oxygen (DO) profile during the cycle. Multiple stable periods were

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Table 3. Summary of the pilot influent characteristics

Compound	Influent buffer tank (after dilution)	Unit
TSS	1.49 ± 0.77 (n = 122)	$gTSS \cdot L^{-1}$
VSS	0.89 ± 0.52 (n = 122)	$gVSS \cdot L^{-1}$
COD_{Sol}	5.78 ± 1.13 (n = 127)	$gCOD_{Sol} \cdot L^{-1}$
BOD_{other}	0.70 ± 0.07 (n = 117)	$gCOD_{other} \cdot gCOD_{Sol}^{-1}$
VFA	0.50 ± 0.13 (n = 114)	$gCOD_{VFA} \cdot gCOD_{Sol}^{-1}$
Even VFA	0.56 ± 0.12 (n = 114)	$gCOD_{VFA} \cdot gCOD_{VFA}^{-1}$
Odd VFA	0.44 ± 0.12 (n = 114)	$gCOD_{VFA} gCOD_{VFA}^{-1}$
TAN	622 ± 159 (n = 77)	$mgN \cdot L^{-1}$
Phosphate	20.9 ± 10.8 (n = 77)	$mgPO_4\text{-}P \cdot L^{-1}$
pH	7.53 ± 0.42 (online data August 2017)	—
Alkalinity	70 ± 10	$meq \cdot L^{-1}$

Note: Here TAN represents the total ammonia nitrogen, which was ammonia (NH_3) + ammonium (NH_4^+). The alkalinity of the substrate was estimated to be around 70 $meq \cdot L^{-1}$ using the measured concentrations of TAN and VFA and estimating a pCO_2 of 9% (see the Appendix).

observed during the operation of the PHA producing pilot plant. A stable period comprised all cycles with a feast length below 50 min with the condition that the standard deviation of the feast length of the period including the surrounding 3 days (7 days in total) was below 15 min. Furthermore, specific days were included that would not pass the qualifications due to, for example, technical difficulties. An overview of the stable periods is given in Fig. 2.

In this study, we describe in detail the period from July 22, 2017, to January 26, 2018 (188 operational days). During this period, the operational settings of the systems were kept constant, providing a representative overview of the system performance.

During this period, a DO pattern typical for the enrichment of PHA producing microorganisms using a feast-famine regime was observed. This typical pattern consisted of two phases; the first phase, called the feast phase, in which a high oxygen respiration rate was observed; and the second phase, called the famine phase, in which the oxygen respiration rate was lower (Fig. 3, operational day: August 29, 2017).

The pH dropped during the feeding phase (duration: 16 min); once the feeding was completed, the pH gradually increased due to VFA uptake. The system characteristics of the enrichment reactor are summarized in Table 4.

During the settling phase, the DO dropped to zero, and all settleable biomass settled to the bottom part of the reactor. After the settling phase and removal of the supernatant, the aeration was turned on again, entering the famine phase. The DO was slowly increased in this phase, indicating the depletion of the intracellular PHA; non-PHA VSS increased concurrently, indicating growth. The pH in this phase was also slowly increasing from 8.5 to 9 likely due to the stripping of CO_2.

Microbial Community Analysis

FISH microscopy was used to investigate microscopically the diversity of the enriched culture. Initial 16S rRNA (NGS) analysis indicated that an unknown species was present in theenrichment. A specific probe targeting the *uncultured Rhodocyclaceae bacterium clone JT01* was designed, as described in the Material and Methods section. Three different probes for FISH analysis used were EUB338I-III (Cy5) to bind to all prokaryotic bacteria, UCB823 (Fluos) to bind to *P. acidivorans*, and JT01 (Cy3) to target clone JT01. Over the period described in this study, multiple FISH slides were prepared, and in all of them, clone JT01 seemed to be the dominating microorganism in the enrichment. The bacteria appeared to be a large betaproteobacteria, and sizes varied and

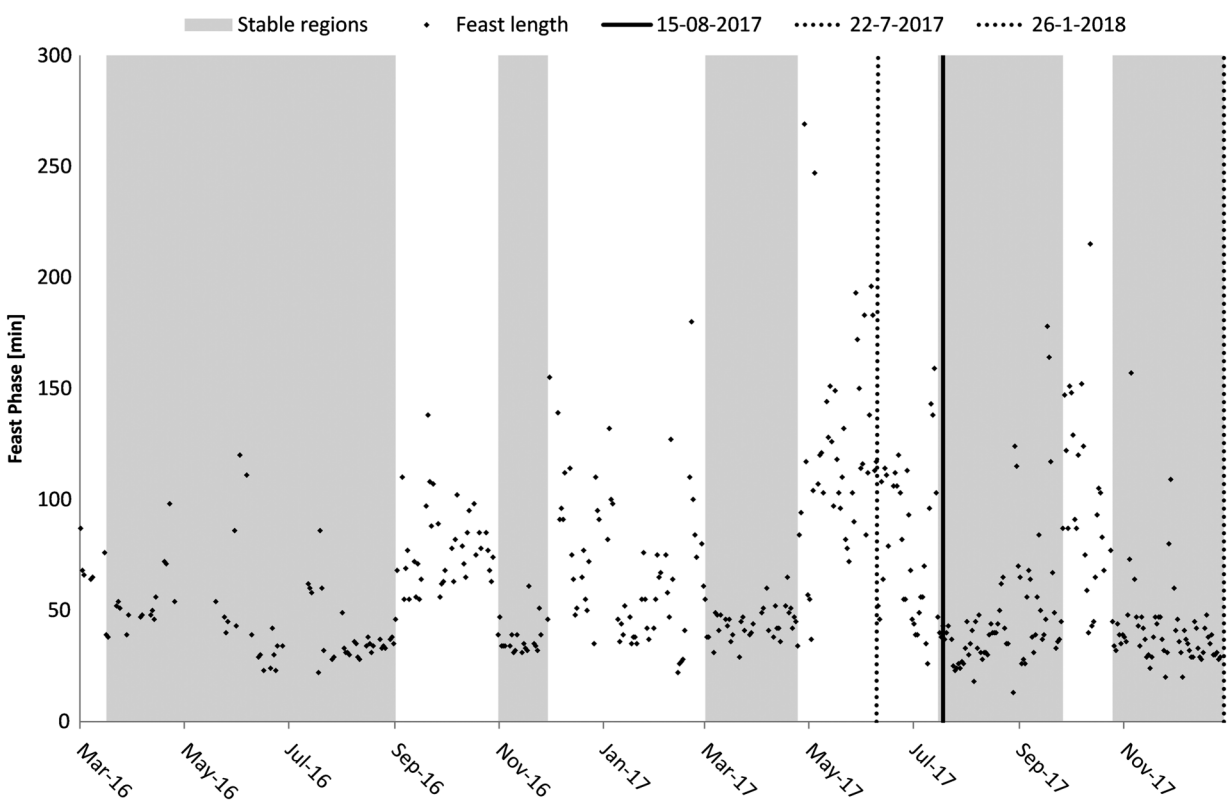

Fig. 2. Overview of the performance of the enrichment reactor in terms of the length of the feast phase.

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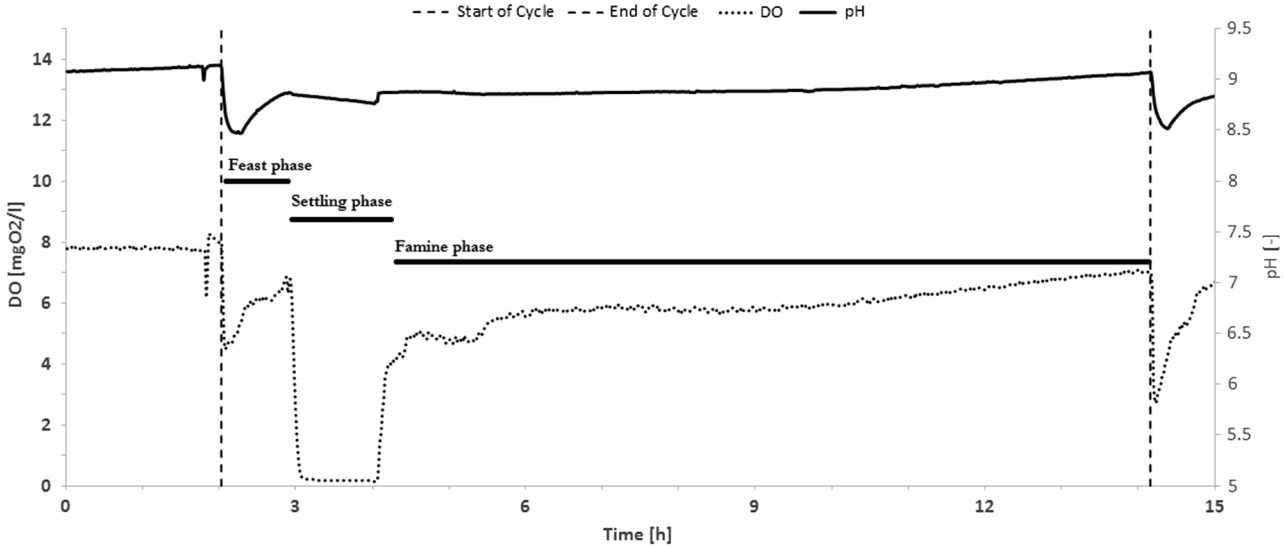

Fig. 3. Dissolved oxygen profile and pH profile in the enrichment reactor in a stable period, from operational day July 29, 2017.

Table 4. Characteristics of the enrichment reactor averaged over time

Compound	Enrichment reactor	Unit
PHA (end of cycle)	0.04 ± 0.03 (n = 27)	$gPHA \cdot gTSS^{-1}$
TSS (end of cycle)	4.56 ± 1.57 (n = 119)	$gTSS \cdot L^{-1}$
VSS (end of cycle)	3.45 ± 1.16 (n = 118)	$gVSS \cdot L^{-1}$
SVI30 (end of feast)	66 ± 38 (n = 120)	$mL \cdot gTSS^{-1}$
PHA (end of feast)	0.26 ± 0.09 (n = 109)	$gPHA \cdot gTSS^{-1}$
TSS (end of feast)	3.48 ± 1.12 (n = 121)	$gTSS \cdot L^{-1}$
VSS (end of feast)	2.64 ± 0.84 (n = 120)	$gVSS \cdot L^{-1}$
q_{VA}	4.2 ± 1.9 (n = 108)	$gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$
q^*_{VFA}	2.4 ± 1.2 (n = 108)	$gCOD_{VFA} \cdot gVSS^{-1} \cdot h^{-1}$
Y^{PHA}_{VFA}	0.44 ± 0.14	$gPHA \cdot gCOD_{VFA}^{-1}$
Y_{sCOD}^{X}	0.25 ± 0.08	$gVSS \cdot gCOD_{Sol}^{-1}$

bacteria as large as 3–5 μm were observed. The bacteria species belonged to a yet unknown species and genus, and the closest related species was the *Thauera* genus, as shown in Fig. 4.

Accumulation

To assess the PHA accumulation potential of the enrichment, batch experiments were performed. The experiments were started with a small inoculum containing 10 L of mixed effluent from the enrichment reactor (containing 33 gVSS) and 170 L of influent containing on average 440 $gCOD_{VFA}$. The average COD_{VFA}:P:N ratio was 1:4:108 ($gCOD_{VFA}$:mgP:mgN), suggesting that the accumulations were performed in the presence of nutrients. Data obtained during an accumulation experiment conducted on August 15, 2017, are shown in Fig. 5 as an example. In this experiment, virtually all VFAs were consumed in less than 3 h, and a PHA content of $0.61\ gPHA \cdot gVSS^{-1}$ was reached. The polyhydroxybutyrate (PHB) content was $0.43\ gPHB \cdot gVSS^{-1}$, and the polyhydroxyvalerate (PHV) content was $0.18\ gPHV \cdot gVSS^{-1}$.

There were two mechanisms assumed for nonPHA VSS contribution in the process: (1) inert particulate solids in the influent, and (2) biomass growth in the accumulation bioreactor. The initial increase in VSS represents the inert VSS part of the influent added while filling up the reactor and the simultaneous production of PHA. After the feeding was finished, a constant PHA production rate was observed, increasing VSS. Determining the X_{PHA} present in the system, as described in the Material and Methods section, a cellular PHA content of $0.86\ gPHA \cdot X_{PHA}^{-1}$ was estimated. Furthermore, the COD consumption during the accumulation period was 95% because of the consumption of VFAs; thus, almost no BOD_{other} was consumed. Process characteristics were obtained from the measured data to interpret the performance of the system, and these are summarized in Table 5. The specific uptake rates for the PHA producer (q_{VFA}) obtained in the accumulation were higher than those obtained during the cycle characterization.

Over the period described in this study, multiple accumulations were performed. The enrichment was exposed to the substrate for at least 3 h and subsequently settled for 30 min prior to sampling, and a maximum amount of $0.88\ gPHA \cdot gVSS^{-1}$ was measured. On average, a PHA content of $0.61 \pm 0.14\ gPHA \cdot gTSS^{-1}$ (n = 7) expressed per TSS and $0.77 \pm 0.18\ PHA \cdot gVSS^{-1}$ (n = 3) expressed per VSS was obtained. The PHB content was on average $0.38 \pm 0.09\ gPHB \cdot gVSS^{-1}$ (n = 3), and the PHV content was on average $0.38 \pm 0.09\ gPHV \cdot gVSS^{-1}$ (n = 3).

Discussion

Enrichment Performance

The microbial community established in this work was able to consume the supplied VFAs with an estimated PHA specific biomass substrate uptake rate of $4.2 \pm 1.9\ gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$ (n = 108). This q_{VFA} was in a range comparable to lab studies (Albuquerque et al. 2011; Jiang et al. 2011; Johnson et al. 2009; Marang et al. 2013). In lab studies, biomass maximum specific uptake rates of $5.6\ gCOD\ gX_{PHA}^{-1} \cdot h^{-1}$ were reported for cultures enriched using acetate and $10.6\ gCOD\ gX_{PHA}^{-1} \cdot h^{-1}$ for cultures enriched on butyrate (assuming $C_1H_{1.8}O_{0.5}N_{0.2}$ corresponding to $25\ gX \cdot cmolX^{-1}$) (Beun et al. 2002; Jiang et al. 2011; Marang et al. 2013). In this study, a mixture of different linear VFAs was present, and of which, acetate was the most abundant $0.44 \pm 0.11\ gCOD_{Ace} \cdot gCOD_{VFA}^{-1}$.

This study also showed the importance of estimating the PHA producing culture as a fraction of the VSS in order to estimate a qS value. The q^*_{VFA} was $2.4 \pm 1.2\ gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$ (n = 108), which was almost 50% lower than the q_{VFA}. The data

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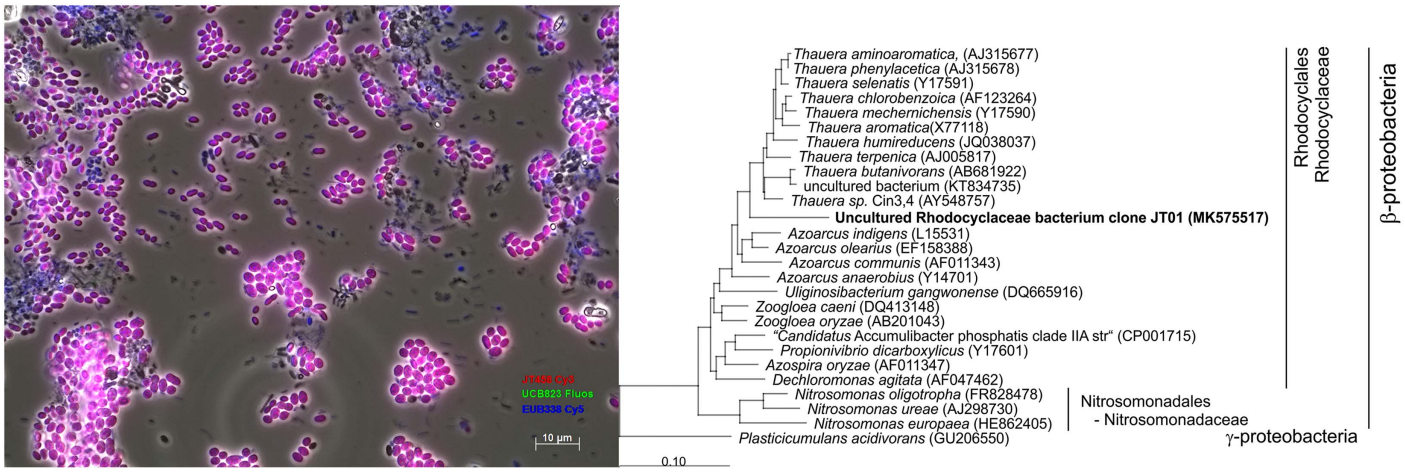

Fig. 4. FISH microscopy picture of the enriched culture on diluted leachate at pilot-scale, using the following probes: Cy3 JT01, Fluos UCB823, Cy5 EUB 338. This phylogenetic tree was constructed off near full-length 16S-rRNA genes from the clone library and reference database (SSURef_NR99_128_SILVA_07_09_16_opt) using the NeighborHood Joining algorithm implemented in ARB version 5.2. In total, 1,206 positions were taken for the calculation. A position filter, SSU-Ref: bacteria were applied to select for most common positions in the bacterial 16S-rRNA alignment. The scale bar shows a 10% base-pair difference. Two species of the Thermotoga genus were used as the outgroup but later pruned from the tree.

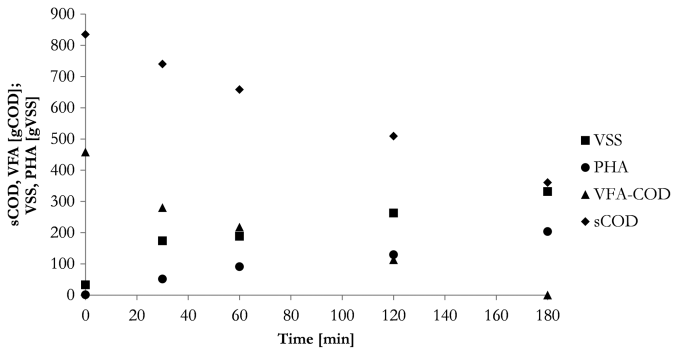

Fig. 5. Detailed characterization of a PHA accumulation potential experiment (August 15, 2017), illustrating the evolution of COD_{Sol}, COD_{VFA}, PHA, and VSS over time in absolute amounts.

Table 5. Characteristics from the PHA producing enrichment exposed to the excess substrate in a batch process

Accumulation reactor		Unit
Y_{VFA}^{PHA}	0.44	$gPHA \cdot gCOD_{VFA}^{-1}$
q_{VFA}	7.5	$gCOD_{VFA} \cdot gX_{PHA}^{-1} \cdot h^{-1}$
q_{VFA}^{*}	4.7	$gCOD_{VFA} \cdot gVSS^{-1} \cdot h^{-1}$
q_{sCOD}^{*}	4.9	$gCOD_{Sol} \cdot gVSS^{-1} \cdot h^{-1}$

suggest that influent solids and nonPHA producing biomass lowered the fraction of PHA producers significantly. The concentration of free ammonia (FA) was around 240 $mgNH_3 - N \cdot L^{-1}$ using the equilibrium constant of NH_3/NH_4^+. This FA concentration was above the inhibition range compared to conventional wastewater treatment inhibition concentrations of FA (Liu et al. 2019). Despite these high FA concentrations, q_{VFA} values obtained in this work were comparable to those observed previously, demonstrating the capacity of the process to adapt to unfavorable environmental conditions.

An overall VSS yield on COD_{Sol} of $Y_{\frac{X}{sCOD}} = 0.25\ gVSS \cdot gCOD_{Sol}^{-1}$ was observed, and this was lower than values reported in the literature for comparable systems: $Y_{\frac{X}{sCOD}} = 0.30\ gVSS \cdot gCOD^{-1}$ (Tamis et al. 2018). The data suggest that the high pH and/or TAN concentration did not significantly affect the biomass specific uptake rate but reduced the efficiency of biomass production. Furthermore, the presence of unstable periods indicated that the process was susceptible to unknown compounds present in the substrate. The leachate of organic waste is such a complex mixture, making it virtually impossible to identify the cause of the unstable periods.

Despite the potentially adverse conditions, the obtained microbial culture appeared highly effective with respect to PHA production. According to the FISH picture, as shown in Fig. 4 the microbial community was dominated by one species. The dominant bacterium was a large betaproteobacteria most closely related to *Thauera Cin3,4*. The species found in the bioreactor had comparably high substrate uptake rates and oxygen respiration rates that are known for the *Plasticicumulans* genus, although both genera are phylogenetically not related. The *uncultured Rhodocyclaceae bacterium clone JT01* described in this work belongs to the betaproteobacteria, family of *Rhodocyclaceae*, whereas *Plasticicumulans* is a gammaproteobacteria. Both bacteria have functional and morphologically similar properties: the bacteria are large, rapid VFA consumers, and can store PHA up to high wt%. The results demonstrate that high respiration rates and high PHA storing capabilities are distributed over different sections of the phylogenetic tree.

PHA production from leachate derived from OFMSW required multiple levels of understanding of the production process. The COD_{Sol} present in the stream contained VFAs that would result in PHA producers, but the stream also contained COD_{Sol}, resulting in nonPHA producers. Furthermore, the leachate contained nonbiodegradable (inert) solids that were divided into settleable solids and nonsettleable solids. The settleable solids were removed by a pretreatment method in which 60% of the solids were removed. The nonsettleable solids entered the reactor with the influent and were observed in comparable concentrations in the reactor effluent suggesting that they were not incorporated into the settling biomass in significant amounts. The fraction of COD_{Sol} consumed by the PHA producer in the bioreactor was on average 82% based upon the VFAs consumed compared to ΔCOD_{Sol}. The impact of BOD_{other}

resulting in the side population being minimized by the introduction of a settling phase shortly after all VFAs were depleted, as proposed in Korkakaki et al. (2016b).

Solid Partitioning

In an operational cycle, multiple phases could be identified that influenced the final composition of the VSS. We assumed that three main components contribute to the final VSS composition: (1) the COD_{VFA} which resulted via the production and conversion of PHA in PHA producing biomass, (2) BOD_{other} consumption resulting in a nonPHA producing side population, and (3) inert VSS present in the substrate. Four time points during a cycle were evaluated (Fig. 6).

The starting point of each cycle (t = 0 h) was after biomass removal at the end of the previous cycle. After t = 0, the reactor was filled with substrate adding COD_{Sol}, nutrients, and inert particulate solids in the system. Next, the culture consumed the VFAs, resulting in VSS production as PHA and PHA producing biomass. On average, 0.82 $gCOD_{VFA} \cdot gCOD^{-1}_{consumed}$ was consumed in the entire cycle, this value was used as $f_{X,PHA}$ (as explained in the Material and Methods section). After all VFAs were depleted (0.8 h), a settling phase was scheduled lasting from 0.8 to 1.8 h. In this phase, solids could settle to the bottom of the bioreactor for 60 min, and after which, the supernatant (86 L) was removed from the system. With the supernatant, at least two compounds were removed from the system: (1) inert solids that were not incorporated in biomass; and (2) half of the slowly degradable BOD_{other} that was not degraded in the feast phase. In the famine phase lasting from 1.8 to 12 h, the PHA was consumed and X_{PHA} was produced. Furthermore, during the famine phase, BOD_{other} was consumed, and this resulted in the production of a side population. The side population ($f_{X,other}$) was calculated using the amount BOD_{other} consumed. Finally, the BOD_{other} that was present in the supernatant could be converted to biomass equivalents using the VSS production yield. This potential biomass removed after settling was classified as prevented side population. In the set-up used in this study, the fraction of PHA producer present in the VSS was 0.56 $gX_{PHA} \cdot gVSS^{-1}$. The portion of PHA producers would have been 0.25 $gX_{PHA} \cdot gVSS^{-1}$ without a settling phase and no pretreatment of the substrate. The higher fraction of PHA producing biomass in the set-up with settling implicates that higher PHA content may be reached: 0.77 $gPHA \cdot gVSS^{-1}$ with the settling versus 0.61 $gPHA \cdot gVSS^{-1}$ without settling.

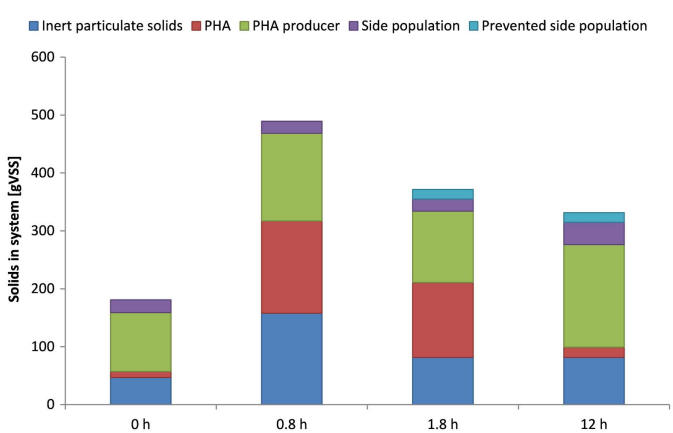

Fig. 6. Solid partition over the cycle. Here, 0 h represents the beginning of the cycle, 0.8 h represents the time point in the cycle when all VFAs were consumed, 1.8 h represents the solid composition in the reactor after 1 h settling and removal of the supernatant. Finally, 12 h represents the end of the cycle.

Accumulation Potential. The PHA accumulation potential of the enrichment was assessed by doing multiple batch experiments to determine the maximum PHA content that could be achieved. One factor that was important enabling the relatively high PHA content was the pretreatment of the influent removing undesired settleable solids. After pretreatment by settling, the substrate still contained 1.5 ± 0.8 $gTSS \cdot L^{-1}$ (n = 122) of nonsettling solids. In the accumulation experiment shown in Fig. 5, this contributed to 30% of the TSS concentration at the end of the accumulation. In several accumulations, it was shown that high apparent PHA wt% such as PHA percentages between 70% and 80% could be established after settling and removal of the supernatant at the end of the accumulation. These experiments showed an additional advantage of the settling technique besides selective removal of the side population. Having a settleable culture made it possible to achieve high apparent PHA wt% in a complex environment containing nonsettling solids.

Generally, achieving more than 0.60 $gPHA \cdot gTSS^{-1}$ and usage of mixed cultures requires defined environments. The PHA content achieved in this study was higher than the PHA content reached on similar leachate in the lab (0.29 $gPHA \cdot gVSS^{-1}$) (Korkakaki et al. 2016a). In Korkakaki et al. (2016a) it was suggested that an unsuccessful enrichment resulted in poor PHA storing capabilities. Remarkably, in the Korkakaki et al. (2016a) study, a culture enriched on the synthetic medium was capable of accumulating up to 0.78 $gPHA \cdot VSS^{-1}$ in batch experiments using a similar type of leachate as substrate. That study performed at pH 7 indicated that the complex medium (in the leachate) in the short term was not significantly inhibiting PHA production, though long-term cultivation on leachate resulted in enrichment of biomass with poor PHA accumulating abilities. This phenomenon of obtaining bad enrichment at prolonged exposure to this complex substrate seemed absent in this study because an enrichment with good PHA accumulating capability was obtained. Possibly, better control of nitrogen and phosphorus availability (by measuring these concentrations daily and making sure that they are not limiting) could explain the difference. This would make sense since the limitation of N or P during enrichment will give a competitive advantage to microorganisms with high N or P uptake rate instead of high PHA production capability (Johnson et al. 2010b).

To reach a high PHA content, a nutrient-limited stream is preferred because of the growth limiting condition created which favors the PHA purity at the end of the accumulation (Johnson et al. 2010a; Korkakaki et al. 2017). PHA storing potentials using streams containing nutrients were generally lower than those obtained with nutrient limitation. A culture enriched on fermented wastewater treatment plant (WWTP) sludge as the substrate for PHA accumulation that was not nutrient limited reached 0.39 $gPHA \cdot gVSS^{-1}$ (Morgan-Sagastume et al. 2015). Leachate, as used in this study, comprised not only conditions where growth was possible, but also other adverse conditions such as a high pH after aeration and containing a high TAN content. Despite these conditions, $0.61 \pm 0.14 gPHA \cdot gTSS^{-1}$ (n = 7) and expressed per organic content 0.77 ± 0.18 $gPHA \cdot gVSS^{-1}$ (n = 3) was obtained, highlighting the importance of having a good inoculum (enrichment).

Industrial Implementation of PHA Production. The production of PHA on different types of wastewater has been investigated on a pilot-scale in several studies, as shown in Table 6. The high PHA contents reported suggesting that PHA producing microorganisms have been effectively enriched on different waste streams. One way of evaluating PHA production from OFMSW was done via the overall PHA production yield. Using an adapted method for obtaining an overall process yield as defined by Bengtsson et al. (2008),

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Table 6. Overview of achieved PHA percentages and PHA production yields on different types of waste streams

PHA percentage ($gPHA \cdot gVSS^{-1}$)	Yield ($kgPHA \cdot kgCOD^{-1}$)	Substrate	Scale	References
0.29	0.07	Leachate of OFMSW	Lab	Korkakaki et al. (2016a)
0.39	—	Fermented WWTP sludge	Pilot	Morgan-Sagastume et al. (2015)
0.46	—	OFMSW-SS mixture	Pilot	Valentino et al. (2019)
0.48	—	Leachate of OFMSW	Lab	Colombo et al. (2017)
0.49	0.11	Fermented organic residues	Pilot	Bengtsson et al. (2017)
0.55	—	Leachate of OFMSW	Pilot	Valentino et al. (2018)
0.76	0.18	Fermented candy bar factory water	Pilot	Tamis et al. (2014)
0.77	**0.14**	**Leachate of OFMSW**	**Pilot**	**This study**
0.80	0.20	Fermented papermill wastewater	Pilot	Tamis et al. (2018)
0.89	—	Synthetic medium	Lab	Johnson et al. (2009)

Note: To highlight the bold value obtained in this study.

the overall PHA production yield on COD_{Sol} in the wastewater was determined. At the Biocel, a VFA-rich stream was present; thus, no pretreatment and COD losses in this step were taken into account for the determination of the overall PHA yield. An overall PHA production yield of 0.14 $kgPHA \cdot kgCOD_{Sol}^{-1}$ was obtained using the following parameters and visualized in Fig. 7:

- A biomass production yield of 0.25 $kgVSS \cdot kgCOD_{VFA}^{-1}$
- A PHA production yield of 0.44 $kgPHA \cdot kgCOD_{VFA}^{-1}$
- A PHA purity at the end of the accumulation of 0.77 $kgPHA \cdot kgVSS^{-1}$ (Table 6)
- A VFA fraction in the substrate of 0.50 $kgCOD_{VFA} \cdot kgCOD_{Sol}^{-1}$

This yield was lower than the PHA production yield obtained in several other studies shown in Table 6. The PHA production yield expressed per $kgCOD_{VFA}$ is much higher and equals 0.29 $kgPHA \cdot kgCOD_{VFA}^{-1}$ because the VFA fraction of the COD_{Sol} was 50%. It should be noted that a large part of the remaining COD was inert COD.

The main upstream operational expenses originate from aeration of the reactors and acid/base dosage (Tamis et al. 2018). This study is a case for potentially eliminating one of these cost factors, namely, the acid/base dosage. For the enrichment and accumulation in this study, no acid or base was used, while both an adequate enrichment of PHA producing biomass and a high PHA content at the end of an accumulation were achieved. A previous study has shown the economical potential of producing PHA from a stream like a leachate instead of biogas (Kleerebezem et al. 2015). One of the key criteria in the economic model is that the operational expenses should be kept low, which can be pursued when pH control is absent. If pH control is not a prerequisite for PHA production from wastewater, the overall process becomes economically more feasible.

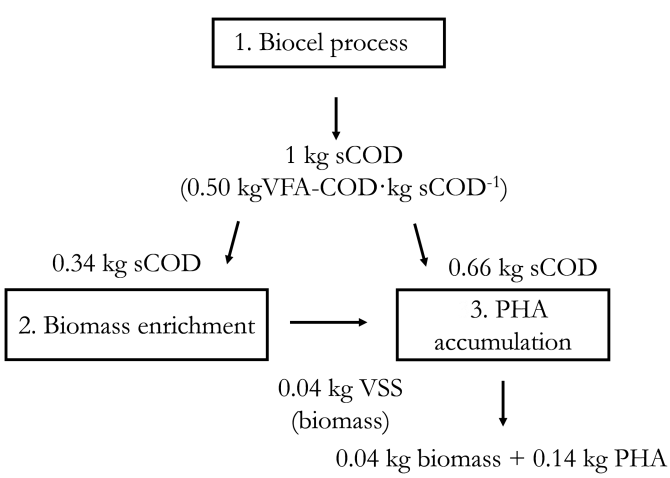

Fig. 7. COD partitioning for the enrichment and accumulation reactor, from 1 $kgCOD_{Sol}$ of leaching product obtained from the Biocel process.

In this study, the substrate used for the pilot plant was diluted 2–3 times with tap water, preventing oxygen limitation in the selector. Besides the COD, other compounds such as ammonium were also diluted. In a full-scale situation, the dilution is done with effluent recirculation of the PHA process if necessary to avoid oxygen limitation. The dilution performed with effluent instead of tap water results in that the ammonia no longer being diluted and likely reaches 1–2 $gN \cdot L^{-1}$. At operational pH-values of 9, these ammonium concentrations may give rise to inhibition. In a full-scale process, nitrogen removal using the anammox process can be implemented to remove (partly) nitrogen and thus alkalinity from the system after the PHA process. The VFA fraction in $kgCOD_{VFA} \cdot kgCOD_{Sol}^{-1}$ was low in this study due to the origin of the substrate from a methanogenic OFMSW digester, implying the effective removal of VFAs by methanogens. Evidently, eventual implementation of the PHA production from OFMSW is anticipated to be based on a hydrolysis pilot that maximizes VFA production and aims for the minimization of methane production. This will evidently increase the overall effectiveness of the PHA production process from OFMSW.

Conclusion

This study shows the successful enrichment of a functional PHA accumulating microbial community grown on OFMSW-leachate without pH control on a pilot-scale. After accumulation for maximization of the cellular PHA content, a maximum PHA content of 0.77 ± 0.18 $gPHA \cdot gVSS^{-1}$ ($n = 3$) was achieved within 3 h of accumulation. Detailed mass balance analysis demonstrated that the actual PHA content in VFA grown cells was highly comparable to previous studies, and the eventual lower PHA content was the resultant from particulates in the influent and growth of nonPHA producers on substrates other than VFAs. This demonstrates the technological feasibility of PHA production in conditions formerly considered unfavorable for PHA production, such as a pH of 8.5–9 and high ammonium concentrations. Herewith, this work contributes to the extension of the operational window of the wastewater based PHA production process and demonstrates that the hoarding strategy based on PHA production is widely distributed in the microbial world.

Appendix. Leachate Alkalinity Calculations

The alkalinity of the leachate was estimated using the pCO_2, pH, total TAN, and the known VFA concentration. The pCO_2 in the

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Table 7. $pKpK_a$ values used in this study to estimate the alkalinity of the leachate

pK_a	Chemical reaction
6.38	$H_2CO_3 <--> HCO_3^- + H^+$
10.33	$HCO_3^- <--> CO_3^{2-} + H^+$
4.77	$VFA <--> VFA^- + H^+$
9.30	$NH_4^+ <--> NH_3 + H^+$

digestion tunnels was unknown, and to estimate the pCO_2, the following assumption was made. At the end of a cycle in the enrichment reactor, the pCO_2 was estimated to be 0.1% close to atmospheric values as the broth was aerated for 12 h. At this point in the cycle, the pH reached 9.1. This pH and pCO_2 were used as input for the leachate to obtain the alkalinity of the system. The alkalinity of the system was calculated as follows:

$$Alkalinity = [OH^{-1}] + [VFA^{-1}] + [HCO_3^{-1}] + 2 \cdot [CO_3^{-2}] \quad (12)$$

- Alkalinity was the calculated alkalinity expressed in $meq \cdot L^{-1}$ of the leachate.
- $[OH^{-1}]$ was the concentration of hydroxide (mM) in the leachate.
- $[VFA^{-1}]$ was the averaged concentration of VFAs (mM) in the leachate.
- $[HCO_3^{-1}]$ was the concentration of bicarbonate (mM) in the leachate.
- $[CO_3^{-2}]$ was the concentration of carbonate (mM) in the leachate.

The Henry coefficient for CO_2 (H^{CO2}) used was 2.98×10^{-2} $mol \cdot L^{-1} \cdot atm^{-1}$ for 30°C. The pKa values for each compound contributing to the overall alkalinity are summarized in Table 7.

The alkalinity of the leachate was found to be 70 ± 10 $meq \cdot L^{-1}$. The pH of the substrate was continuously measured when stored in the buffer vessel and was 7.53 ± 0.42. The alkalinity obtained at pH of 9.1 and a pCO_2 of 0.1% should be similar to the alkalinity at a pH of 7.5, as measured in the buffer vessel. Using a pH of 7.5 and an alkalinity of 70 $meq \cdot L^{-1}$, a partial pressure for CO_2 of 9% was found.

Data Availability Statement

All data, models, and code generated or used during the study appear in the published article.

Acknowledgments

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Notation

The following symbols are used in this paper:

BOD_{other} ($gCOD_{other}$) = amount of other biodegradable oxygen demand consumed by the side population;

Feastphase (h) = duration in a cycle when VFAs were present;

f_{XPHA} = fraction of PHA producing biomass;

PHA_{EoC} ($gPHA \cdot L^{-1}$) = PHA concentration at the EoC;

V_{EoC} (L) = volume of the bioreactor at the EoC;

$VSS_{catalytic}$ (gVSS) = amount of catalytic biomass present at the end of cycle (EoC);

VSS_{EoC} ($gVSS \cdot L^{-1}$) = VSS concentration at the EoC;

VSS_{inf} ($gVSS \cdot L^{-1}$) = amount of VSS in the influent buffer tank. The VSS_{inf} was assumed to be inert;

X_{other} (gVSS) = amount of non-PHA producers (side population);

X_{PHA} (gVSS) = amount of PHA producers;

ΔCOD_{sol} ($gCOD_{Sol}$) = amount of COD_{Sol} consumed in a cycle; and

ΔCOD_{VFA} ($gCOD_{VFA}$) = amount of COD_{VFA} consumed in a cycle.

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