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REPORT

Report on optimised PHA production process layout

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List of abbreviations

CDW	Cell dry weight
COD	Chemical oxygen demand
COD_{sol}	Soluble chemical oxygen demand
COD_{tot}	Total chemical oxygen demand
COD_{VFA}	Chemical oxygen demand of the volatile fatty acids
F/C-ratio	Feast/cycle-ratio
FID	Flame ionisation detector
GC	Gas-chromatography
HB	Hydroxybutyrate
HRT	Hydraulic retention time
HV	Hydroxyvalerate
IC	Ion-chromatography
NH_4-N	Ammonia nitrogen
OFMSW	Organic fraction of municipal solid waste
OLR	Organic loading rate
P_{tot}	Total phosphorous
PE	Peoples equivalent
PO_4-P	Ortho-phosphate phosphorous
PHB	Polyhydroxybutyrate
PHA	Polyhydroxyalkanoates
PHV	Polyhydroxyvalerate
SRT	Solid retention time
STP	Sewage treatment plant
TGA	Thermogravimetric analyses
TKN	Total Kjeldahl nitrogen
TS	Total solids
TSS	Total suspended solids
VFA	Volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids
WAS	Waste activated sludge

1 Introduction

Sewage contains valuable substances that can be used as raw materials for biobased products. However, to date this potential has hardly been exploited to its full potential in North-West Europe. This results in loss of valuable materials, CO_2-emmissions and less efficient use of natural resources. The Interreg North-West Europe project WOW! - Wider business Opportunities for raw materials from Wastewater (sewage) - aims to develop three value chains for the recovery of carbon based elements from sewage (see figure 1.1):

The production of biodiesel. The sewage inflow is used to cultivate Microthrix p. which can accumulate lipids. The lipids are extracted, processed and transformed to biodiesel.

The production of bio-oil, biochar and acetic acid. The screening material which mainly consists of cellulose material (toilet paper) is dewatered and dried. In a thermal degradation process (pyrolysis) the dried cellulose material is converted into biochar, bio-oil and acetic acid.

The production of PHA (bioplastic). For this the primary sludge is used. In a biological process, PHA is enriched and extracted. Then the PHA is compounded and processed to an end product.

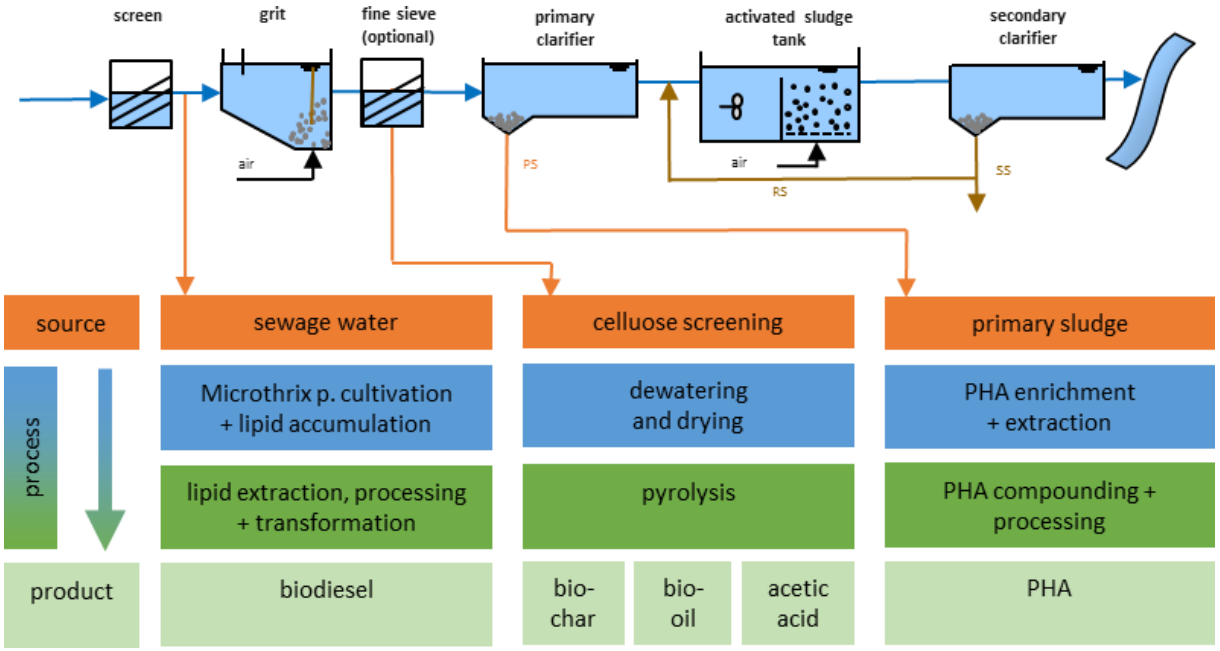


Figure 1.1: Recovery of carbon based elements from sewage in WoW!

One of the main activities of the project was to demonstrate the technical feasibility of these three value chains in three pilots with a focus on optimisation of the different recovery and upcycling techniques and tailoring the products to market needs. This report focusses on the production of Polyhydroxyalkanoates (PHA), a biodegradable polymer using primary sludge as a substrate.

2 Theoretical Background

The production of PHA from sewage sludge and industrial wastewater using waste activated sludge as inoculum is based on a two- resp. three-stage process. As shown in Figure 2-1 the organic compounds in the waste streams are anaerobically converted to short chain volatile fatty acids (VFA) in the first stage. Many microorganisms present in activated sludge can produce PHA from VFA. As the abundance of PHA

producing bacteria is mostly low in activated sludge an aimed enrichment of these bacteria needs to be done in the second stage. By creating a feast/famine-regime with cyclic changing phases of substrate surplus and shortage, PHA-producing bacteria have a selection advantage. During the feast-phase these bacteria produce PHA as an intracellular carbon and energy storage and can fall back on the storage during the famine-phase when no external substrate is present. Other bacteria, which do not have this ability, are driven out over time as their growth is strongly limited by the feast/famine-regime. In the third stage the biomass from the second stage is used in a fed-batch process to maximize the intracellular PHA-content. (Serafim et al. 2004; Dionisi et al. 2007).
After dewatering and drying of the biomass, PHA can be extracted and further processed. For more details on the extraction and processing to a final product see (Persiani et al. 2022).

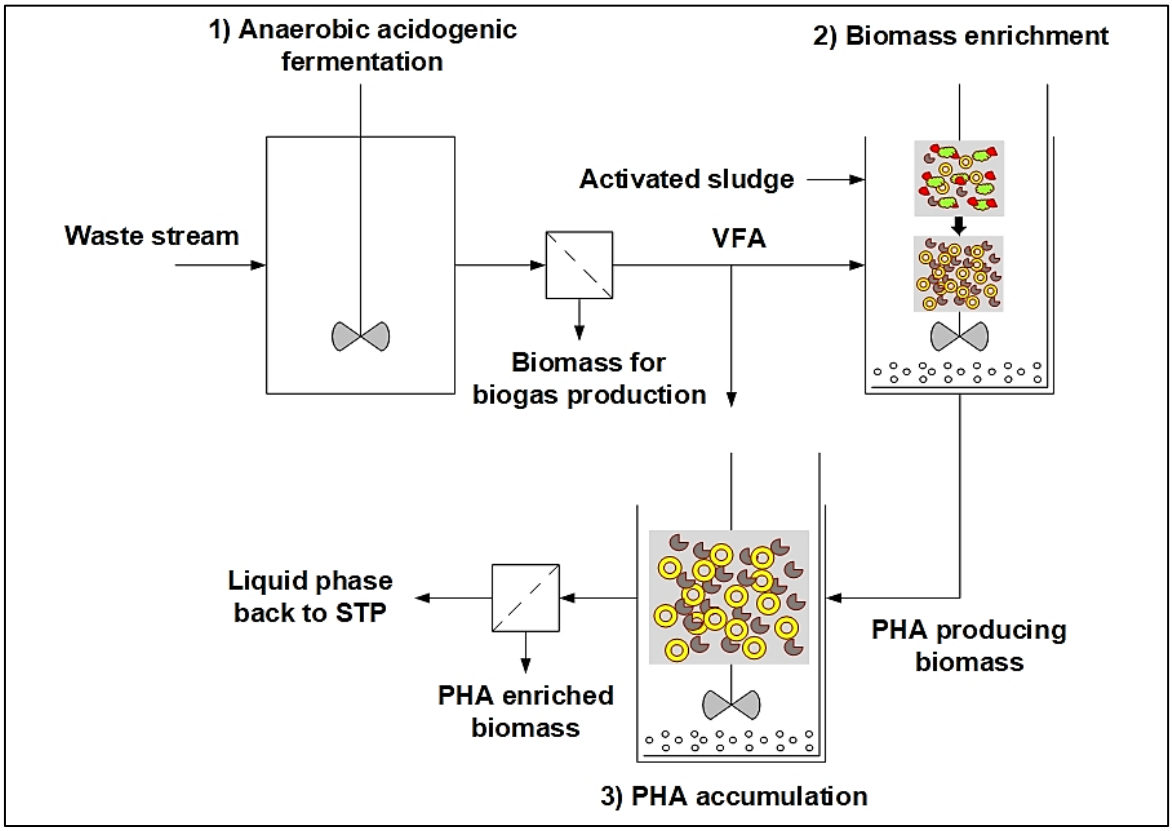


Figure 2-1: PHA-production process adapted from (Pittmann und Steinmetz 2013)

A second approach demonstrated by Werker et al. (2018) is to replace the second stage by a short VFA-acclimation phase. Doing so more VFA are available to produce PHA and are not “wasted” for biomass growth during the biomass enrichment. However, this method requires a waste activated sludge already rich of bacteria with high PHA production potential which is not the case on every sewage treatment plant. For this project the first method was applied.

3 PHA Pilot operational Objectives

The main objective of the pilot operation was to produce PHA solely from material flows of a municipal sewage treatment plant under all seasonal conditions. Furthermore, it was important to demonstrate the possibility to operate the pilot without adding aid flows or chemicals to ensure a sustainable production

process and to minimize operational costs. A special focus was placed on providing notes for practical operating for up-scaling, future operators and planners of PHA production facilities.

4 Materials and Methods

4.1 Operation Site Buchenhofen STP

Wupperverband operates 11 sewage treatment plants with design sizes from 3,750 to 600,000 PE. Buchenhofen STP as the largest plant treats the sewage from the city of Wuppertal and has a current serving size of about 400,000 PE (85 %, COD). The biological reactor has a volume of about 103,000 m³ and is operated as a pre-denitrification process in 6 parallel lines. The biologically treated sewage is finally filtered and discharged to the river Wupper. The produced raw sludge is digested in 3 digesters each with a volume of 6,100 m³. The digester gas is used for combined heat and power production. The central sludge incineration plant (SIP) of the Wupperverband with a capacity of 34,000 Mg DS/a is also located the at the site in Buchenhofen. In the SIP the sludge of the large STPs operated by Wupperverband is combusted together with external sludge from other operators in a fluidized bed incineration. The SIP produces high quantities of excess thermal energy which might be used on site.

4.2 Pilot Installation and experimental Focus

Figure 4-1 shows the process flow diagram with the pilot components.

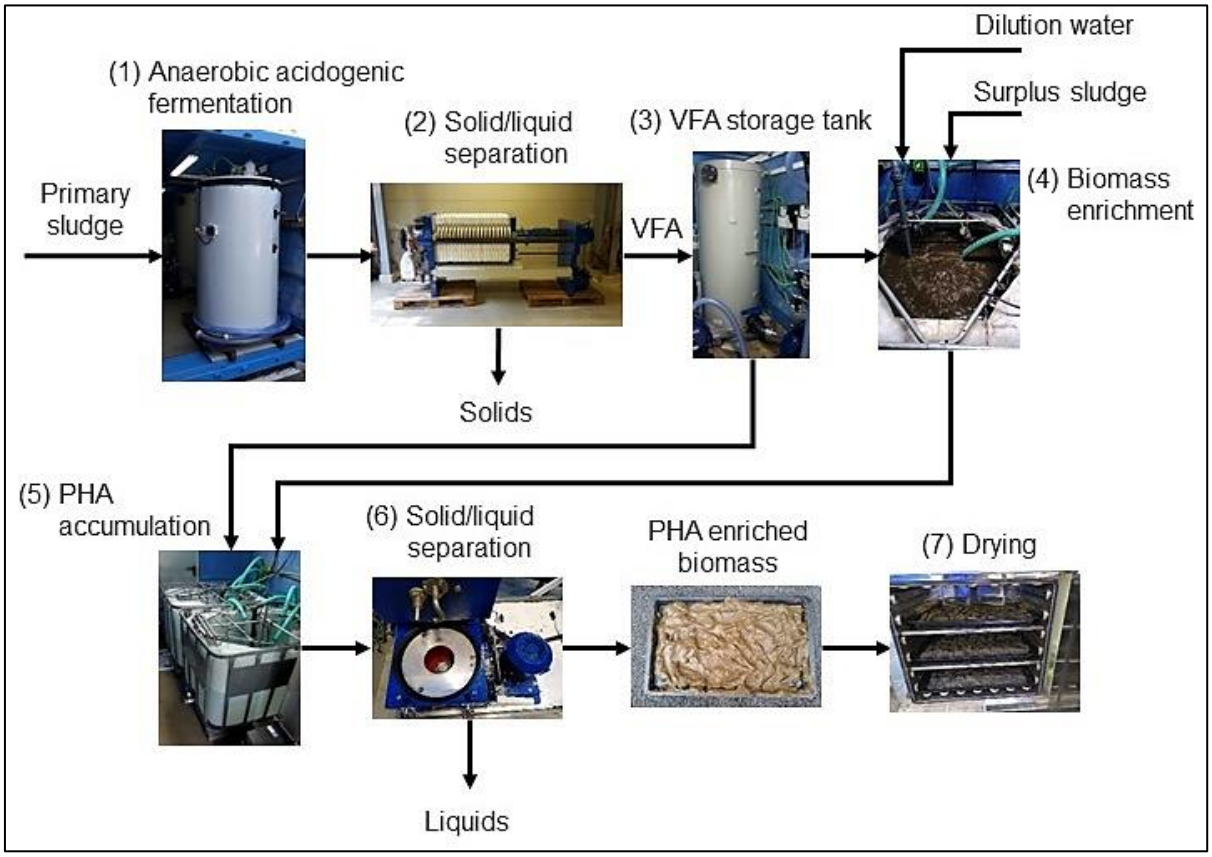


Figure 4-1: PHA-pilot set-up

The pilot consisted of 7 process stages:

1. Anaerobic acidogenic fermentation of primary sludge to produce a VFA-rich stream.
2. Chamber filter press to separate the non-acidified solids from the liquid VFA-stream.
3. VFA-storage tank from where the following process stages are fed.
4. Biomass enrichment tank for selecting biomass with high PHA-production capacities.
5. PHA-accumulation tank for producing biomass with a high intracellular PHA-content.
6. Centrifuge for dewatering the PHA enriched biomass.
7. Drying cabinet for drying the biomass.

In following subsections the process stages are described in more detail.

4.3 Acidification of primary sludge, solid/liquid-separation and VFA-storage tank

The reactor for the anaerobic acidogenic fermentation had a working volume of 1.3 m³ and was fed with primary sludge. The reaction temperature was set to 35 °C and maintained with a heating sleeve (custom-made; PiT). The primary sludge was circulated 2.3 times per hour in the reactor with a peristaltic pump (P_classic 35; Ponndorf Gerätetechnik). The pH was monitored but not controlled. The initial pH was in average 5.8 ± 0.7 and dropped to 4.6 ± 0.6 without any addition of chemicals. The low pH was beneficial to prevent methane production. It should be noted that the anaerobic degradation of proteins forms sulphide. At a pH < 5 in the reactor the chemical balance shifts completely to H_2S (Castro Neto et al. 2018) whereby safety measures should be applied. Lab pre-tests showed that a precipitation with iron salts to form iron sulphide was not possible in this case as iron sulphide redissolved at pH-values < 6. At pH-values ≥ 6 a H_2S-foramtion could be prevented but instead a methane formation occurred (qualitative measurements) what should be prevented for the pilot operation. As the formed total amount of H_2S in the pilot plant was relatively low compared to a full-scale plant an exhaust air system with a radial ventilator (C 30/4 T ATEX; Vortice) was sufficient to prevent a H_2S accumulation in and around the pilot.

The retention time was set to 6-7 days for an additional prevention of a methane production and to allow a weekly working routine. After 6-7 days the reactor was emptied, and the content was pumped into a mixing tank prior to solid/liquid-separation. A chamber filter press (630 x 630 mm plates; Hakü) with polyamide filter cloths (500 L/(dm² min) air permeability) fed with a compressed air diaphragm pump (AH 25; Almatec) was used for the separation. Lab pre-tests showed that the acidified primary sludge was poorly filterable. It was aspired to keep the solid/liquid-separation as simple as possible to be able to investigate if the process would work with little technical effort and thereby reduce investment costs for full-scale plants. It was also tried to avoid adding conditioning agents to keep the VFA-feed preferably pure. E.g., polymers might leave COD-based residuals and would increase the costs for a later process, lime milk would increase the pH-value too much for a usage in the PHA-process. A decanter centrifuge probably would have been a promising alternative device for solid/liquid-separation but couldn't be afforded because of tight budgeting, therefore an improvised solution had to be implemented. By using a filter aid consisting of a mixture of cellulose and perlite (VarioFluxx® P; Erbslöh Geisenheim) usually used in winemaking, a satisfactory filtration was achieved without altering the properties of the VFA-feed. A COD_{sol} determination prior and after the filtration confirmed that no COD was leaked into the VFA-feed by the filter aid ($COD_{sol,pre}/COD_{sol,after}$ = 1.0 ± 0.1; n = 7). The filter aid was only used to be able to continue with the pilot operation and should not be considered as a viable solution for full-scale plants due to procurement costs. Pittmann (2015) showed that the remaining COD in the solid part of the acidified primary sludge can be used for producing biogas (for potential see also (Abels 2019) cited by (WIW 2020)). Hence it seems obvious

to keep the solids after solid/liquid-separation pumpable for feeding into an anaerobic digester, which indicates the use of a decanter centrifuge in full-scale plants.

After filtration, the VFA-rich solution was pumped into a storage tank before using it for the biomass enrichment and PHA accumulation. The retention time of the VFA in the storage tank was about 7 days. Analyses after filling the tank and 7 days later showed that the VFA-concentration was stable and no loss occurred during storage ($COD_{VFA,0\ days}/COD_{VFA,7\ days}$ = 1.0 ± 0.0; n = 8).

The experimental focus of the acidification was to examine the composition of acidified primary sludge over an all-seasonal operation. For an up-scaled operation it is important to know if parameters like the VFA-concentration or nutrient composition vary, as the mode of operation for the following process steps might need to be adapted. Furthermore, it is essential that the VFA-compositions stays stable as this influences the PHA-composition. For the final processing and the product application the PHA-composition needs to remain stable.

4.4 Biomass enrichment, PHA-accumulation, dewatering and drying

The reactors for biomass enrichment and PHA accumulation were largely identically built. The tanks were operated with varying reaction volumes of 200-800 L depending on the operation phase. They were in each case aerated and mixed with a compressor unit (LAM-200; NITTO KOHKI) and two fine-bubble membrane plate diffusers (OXYFLEX®; Supratec). An additional mixing unit was not necessary. The airflow was manually adjusted to prevent oxygen limitation during all reaction states, especially during the feast-phase and PHA accumulation (airflow up to 200 L/min). pH was monitored but not controlled. As it was one goal to examine the feasibility of producing PHA even under low temperatures the tanks were not heated.

The biomass enrichment was operated in a 12 h cycle feast and famine regime. Every 12 h a VFA-feed was pumped into the reactor with a peristaltic pump (Verderflex Rapide R8, Verder) and introduced the feast-phase (usually 1-3 h). To achieve the desired organic loading rate (OLR) and hydraulic retention time (HRT) process water from the river Wupper was used for dilution (pumped with Oxylift 2; Jung Pumpen). Effluent from a STP could be an alternative to avoid using tap water. Before every second VFA-feed a sedimentation phase was implemented to retain biomass. Therefore, after 12 h either the mixed liquor or the supernatant was pumped out (Oxylift 2; Jung Pumpen). The solid retention time (SRT) and HRT were set to 4 d resp. 2 d for three runs and in a fourth run to 2 d and 1 d. The OLR of the first three runs was 0.8-1.4 g COD_{tot}/(L d) (0.5-0.8 g COD_{VFA}/(L d)) and in the fourth run 2.7-2.9 g COD_{tot}/(L d) (2.1-2.3 g COD_{VFA}/(L d)). Table 4-1 summarizes all the runs.

During the first biomass enrichment experiments it was observed that the total solid content (TSS) in the tank dropped under 1 g/L. To increase the TSS, and thereby the total amount of possible amount of harvested biomass, a second variant was tested in which a sedimentation phase was implemented before every end of the cycle and after 6 d the complete reactor content was used for the accumulation. The SRT was 6 d, the HRT 2 d and the OLR in three cases 2.2-2.6 g COD_{tot}/(L d) (1.5-1.8 g COD_{VFA}/(L d)) and in on case 1.1 g COD_{tot}/(L d) (0.5 g COD_{VFA}/(L d)).

Table 4-1: Process parameters of biomass enrichment runs

Number of runs	OLR (COD_{tot})	OLR (COD_{VFA})	SRT	HRT
-	in g/(L d)	in g/(L d)	in d	in d
3	0.8-1.4	0.5-0.8	4	2
1	2.7-2.9	2.1-2.3	2	1
3	2.2-2.6	1.5-1.8	6	2
1	1.1	0.5	6	2

Once a week the drained mixed liquor from the biomass enrichment was used for PHA-accumulation. The accumulation was operated as a fed-batch reactor wherein over 24 h every 30 min a VFA-feed was pumped into the reactor (Verderflex Rapide R8; Verder). One feed consisted of about 100 mg VFA/(L starting volume). The 24 h duration of the accumulation was chosen for a better workability. Once the accumulation was finished, the untreated mixed liquor was pumped (Oxylift 2; Jung Pumpen) to an intermediate tank prior to dewatering. During the first phase of the pilot operation, it was tried to dewater the biomass with the same chamber filter press described in chapter 4.3. As the pure biomass was not filterable, perlite as an inorganic filter aid was used but because of the increased total mass an extraction would not have been possible anymore (see also (Persiani et al. 2022)). Therefore, a basket centrifuge (ZS21 EUR; Eurotec Innovation) with a 3.5 L basket and 2070 g was used instead during the second phase. The dewatered biomass was dried at 80 °C for 48 h in a drying cabinet (U110; Memmert).

The focus of the biomass enrichment and PHA-accumulation was to study an all-seasonal operation without temperature control, solely fed with a VFA-solution produced from primary sludge and without adding nutrients or other chemicals e.g., for pH-control or PHA-stabilization after the accumulation. To gain more knowledge for a process understanding, samples for DNA-analyses from the enrichment tank and from the VFA-storage tank were taken regularly (see chapter 4.7.4 and 5.3).

4.5 PHA-stability after accumulation

For extracting PHA from biomass with di-methyl-carbonate the biomass must be dewatered and dried (see (Persiani et al. 2022)). As both process steps can last several hours, a PHA degradation may occur. Therefore, an inactivation of microbiological activity is usually done in lab- and pilot-scale studies. Werker et al. (2013) proposed to lower the pH to 2-5 e.g. with H_2SO_4 to stop a PHA degradation. Lorini et al. (2021) could demonstrate that a thermal treatment (145 °C for 30 min) of the centrifuged biomass can reliably stabilize the PHA-content. Both methods are indeed suited to prevent a PHA loss during down-streaming, but the use of external chemicals and the energy input for a thermal shock might increase the finale product price and could stand against a sustainable process development.

To evaluate the magnitude of PHA loss prior to dewatering and drying and to develop strategies for the down-streaming after the accumulation, PHA degradation tests were done. Two sets of grab samples were taken at the end of an accumulation batch and one set after the dewatering step. The first two sets consisted of mixed liquor form the PHA-accumulation tank, in the second set VFAs from the acidified primary sludge were additionally added to stabilize the PHA. By using this internal process flow no additional costs would occur and the supernatant after dewatering could be recycled into the biomass enrichment again. With a mixture of ¼ of VFA (pH ca. 4.9-5.0) and ¾ of sample volume (pH ca. 7.5-8.0) a pH of 5.5 was achieved. The samples were not aerated or mixed to mimic a sedimentation tank prior to the dewatering step. The third set which consisted of dewatered sludge was taken after centrifugation and left untreated. After defined times (up to 6 h) the samples were inactivated and freeze dried (see chapter 4.7.3).

To further evaluate the causes for PHA-losses additional samples were taken after various process steps and were differently treated. Besides the mentioned time until dewatering and drying, the drying step itself could cause two effects for PHA-loss. The first effect is the time until the biomass is completely dried in the oven. The second effect is caused by a worse extractability of oven dried samples, probably because of an unfavourable extraction surface due to the chosen drying process. To examine potential PHA-losses of the mentioned causes, sludge samples were taken after centrifugation. The first one was left untreated and dried at 80 °C for 48 h, the second was resuspended in water and inactivated by adding sulfuric acid to a pH-value under 2, centrifuged again (see chapter 4.7.3) and also oven dried, the third sample was inactivated like the previous one but freeze dried instead. Three combinations were closer examined by comparing the PHA-content to a sample taken directly after the accumulation and among the sludge samples (see Table 4-2).

Table 4-2: Sample treatments and examined combinations to evaluate potential PHA-losses during dewatering and drying

Examined sample	Reference sample	Cause of potential loss
Untreated and oven dried	Inactivated after accumulation	Time until dewatering and oven drying (time and extractability)
Dewatered, acidified and oven dried	Dewatered, acidified and freeze dried	Oven drying (extractability)
Untreated and oven dried	Dewatered, acidified and oven dried	Oven drying (time)

4.6 Trace pollutions

As it is known that sewage streams contain trace pollutions such as pharmaceuticals it is important to know if those substances are washed out or eliminated during the process or could be still in the end product. Besides the analyses of the end product, analyses of the solid intermediate products (PHA enriched biomass or extracted raw PHA) could be done to examine if there are trace pollutions present which could remain in the process chain.

The cooperating lab of the Wupperverband examines traditionally trace pollutions only in filtered liquid streams. Therefore, it was not possible to analyse the solid samples. Nevertheless, were for a first orientation random samples taken from some process flows. By that it was tried to determine if a trace pollution accumulation in the PHA enriched biomass could be expected.

Samples were taken during the PHA accumulation with the biomass from the enrichment runs without sludge withdrawal (SRT = 6 d and HRT = 2 d). The starting volume for the accumulation batch could not be analysed therefore was the starting trace pollution mass calculated. Assuming that after 6 d of biomass enrichment with 2 d HRT none of the trace pollution from the inoculum were still present in the liquid phase, the starting trace pollution mass was calculated with a simplified mass balance with the analyses from the VFA-feed and the dilution water and their ratio of the feed. The trace pollutions added during the accumulation were calculated by a mass balance of the VFA-feed. The sum was compared with the mass balance of the centrate after dewatering.
Analysed parameters were Carbamazepin, Diclofenac, Metoprolol, 1H-Benzotriazol and 4 resp. 5-Methyl-1H-Benzotriazol.

4.7 Analytical Methods

4.7.1 Standard urban water management parameter

Standard urban water management parameters were measured either according to standard methods or with Hach cuvette tests. Table 4-3 summarizes the applied methods.

Table 4-3: Applied methods for determining standard urban water management parameter

Parameter	Method	Remarks
COD_{tot}	DIN 38409-41	-
COD_{sol}	Photometric quick test	Hach cuvettes; LCK 514
TKN	DIN EN 25663	-
NH_4-N	Photometric quick test	Hach cuvettes; LCK 303 or 304
P_{tot}	DIN EN ISO 6878	-
PO_4-P	Photometric quick test	Hach cuvettes; LCK 349 or 350
TSS and VSS	DIN 38 409- H 2-2	Black ribbon filter; 589/1, D = 100 mm, Fa. Whatman
TS	DIN EN 15934	-
VS	DIN EN 15935	-

To remove solids for the analyses of COD_{sol}, NH_4-N and PO_4-P samples were centrifuged for 10 min at 4430 g (Z 206 A; Hermle) and two-step filtrated through a folded filter (595½, D = 185 mm; Whatman) and a syringe filter (0.45 µm, Minisart, regenerated cellulose, D = 25 mm; Sartorius).

4.7.2 Volatile fatty acids

For a rough but quick assessment of the volatile fatty acid (VFA)-concentration, samples were directly measured with Hach cuvette tests (LCK 365). The more precise measurement of the VFA-concentration and -composition was done by ion chromatography (930 Compact IC Flex with Metrosep Organic Acids - 250/7.8 cation exchange column; Metrohm). The samples were analysed for formic-, lactic-, acetic-, propionic-, butyric-, iso-butyric-, valeric-, iso-valeric- and caproic-acid. Solid removal was done like described in chapter 4.2.1.

The IC-measurements were conducted in Kaiserslautern about 3 months after the samples were taken and therefore preserved by adding sulphuric acids to drop the pH under 2 and frozen at -20 °C. By using this method a preservation up to 6 months is possible (van Loosdrecht et al. 2016). Every other 3 months the samples were sent to Kaiserslautern in a dry ice cooled parcel.

4.7.3 PHA

For evaluating the pilot plant operation, grab samples (~400 mL, referred to as analytical samples) were taken directly after each accumulation batch, acidified with sulphuric acid to a pH under 2 for inactivation of the microorganisms and prevent a potential PHA degradation. The harvested PHA enriched biomass for the

following process chain (referred to as pilot samples) was not acidified to maintain a PHA production process without chemical addition.

The analytical samples were centrifuged at 4430 g for 1 min (Z 206 A; Hermle) and the supernatant was discarded. Afterwards the samples were washed with deionized water and centrifuged again, which was repeated three times. Afterwards the analytical samples were frozen at -20 °C until a dry ice cooled shipping of the samples to Kaiserslautern for freeze-drying (VaCo 5; ZIRBUS technology GmbH) and analyses.

PHA-content and polymer composition were determined at least in duplicates according to (Braunegg et al. 1978). Approximately 5 mg of the sample was weighed into a 9 mL centrifuge DURAN®-glas tube (16 x 100 mm, conical bottom, PTFE coated GL 18 cap, Rettberg) and mixed with 1 ml of a methanol solution (LiChrosolv®, Merck KGaA) containing 5 % sulfuric acid, 0.8 ml of chloroform (>99 %, Fisher Scientific) and 0.2 ml of benzoic acid (99.5 %, Acros Organics) dissolved in chloroform (5 mg/ml) as internal standard. Subsequently, the samples were heated in a thermo shaker (MHR 23, Hettich) for 6 h at 100 °C with continuous shaking at 450 rpm. After cooling to room temperature, 1 ml of 1 % NaCl solution (>99.5 %, Carl Roth GmbH + Co. KG, in ultrapure water) was added and samples were first shaken to promote phase separation and then centrifuged at 4500 g for 5 min (SORWALL LYNX 600; Thermo Fisher Scientific™). This was followed by extraction of the lower solvent phase using a 1 ml-syringe. The solvent phase was measured using a GC-FID (Agilent 8860 design; Agilent Technologies™) with a HP 5 column (30 m x 0.32 mm, 0.2 µm), an injection volume of 0.5 µl, a flow rate of 20 ml/min helium, a split ratio of 1:50 with nitrogen. Detector temperature was set to 250 °C with a heating rate of 10 °C/min after an initial temperature holding phase of 60 °C over 4 min. Evaluation was based on calibration using a PHBV standard (poly(3-hydroxybutyric aicd-co-3-hydroxyvaleric acid), 8 % PHV-content; Sigma-Aldrich Co.) and an internal benzoic acid standard (see above).

The pilot samples were dried at 80 °C for 48 h in a drying cabinet and sent to Kaiserslautern. Parts of the pilot samples were grinded with a pestle and mortar to increase the extraction surface and analysed as described above.

A second set of analytical and pilot samples were sent to Avans University for thermogravimetrical analyses (TGA) (described in (Persiani et al. 2022)) for comparison (TGA vs. GC-FID). For the comparison was the absolute value for the differences in each case calculated and averaged. The difference in the PHA-content (PHA/TSS) was for the analytical samples (7 ± 5) % (n = 16) and for the pilot samples (3 ± 2) % (n = 6) without a clear tendency of an over- or underestimation of one of the methods. The causes for these differences could not be fully cleared and must be further investigated. In this report were the PHA-contents used determined by GC-FID and referred to VSS.

The extraction for developing the process chain is described in (Persiani et al. 2022).

4.7.4 DNA

For better understanding of the PHA production process and to gain knowledge for a process optimization, samples of acidified primary sludge, of the VFA-storage tank and as well as of the PHA-producing microbial community were taken regularly from the substrate tank and the enrichment reactor of the pilot plant. The samples were subsequently frozen and stored at -20 °C before transport to Kaiserslautern (see chapter 4.7.2). Before further processing, these samples were thawed, centrifuged for 10 min at 4000 g (Varifuge 3.0, Sorvall LYNX 6000; Thermo Fischer) and the supernatant was discarded. Afterwards, total genomic DNA was extracted from each sample with the DNeasy PowerSoil Kit (QIAGEN). For this, the manufacturer's protocol was followed with one exception; instead of 0.25 g 1 ml of sample was used. After

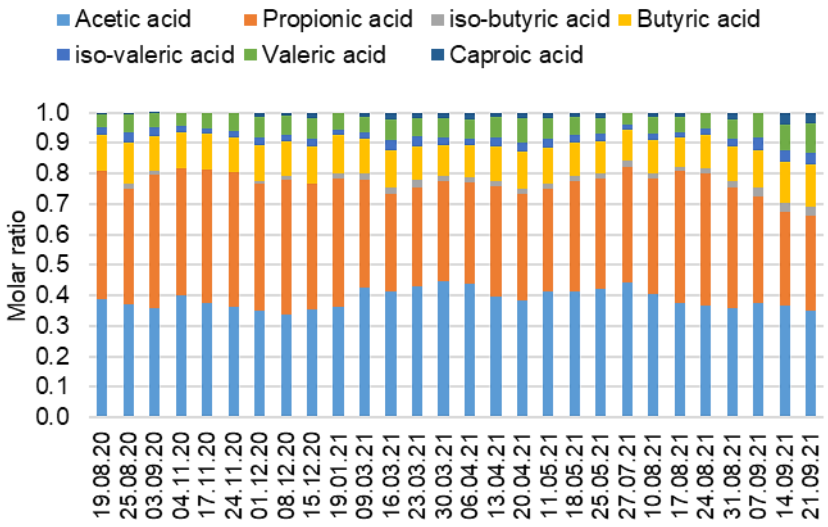
verifying the quality and quantity of extracted DNA by nanodrop measurement (Nanodrop™ 2000, Thermo Fisher Scientific™; Waltham), samples were sent to an external company for PCR (“polymerase chain reaction”) amplification and sequencing of the V3-V4 region of the bacterial 16S-rDNA via Illumina Miseq (Illumina Inc.). After quality control of the raw sequences and bioinformatic analysis using the DADA2 workflow (Callahan et al. 2016), the ASVs ("amplicon sequence variants") were taxonomically assigned using the Greengenes database (DeSantis et al. 2006). The following biostatistical analysis was done with RStudio (Version 4.1.0, RStudio Team, Boston, Massachusetts, USA).

5 Results and Discussion

5.1 Acidification of primary sludge

During the pilot operation 31 batches of primary sludge acidification were successfully performed. The main evaluation parameters for the acidification are the VFA-composition, which influences the PHA-properties, the VFA-yield and the achievable VFA-concentration as they determine the PHA production potential. An additional factor is the nutrient composition. In the following, all parameters are displayed and conclusions for an up-scaled process are discussed.

Figure 5-1 shows the VFA-composition and the range of each volatile fatty acid of all 31 acidification batches. Noteworthy is that the VFA composition showed no tendency of a seasonal influence, as a matter of fact the composition in all batches was relatively stable. It is known that the VFA-composition directly influences the PHA-composition. VFAs with an even number of carbon atoms (acetic, butyric, isobutyric, caproic acid) tend to be processed to PHB and with an odd number (propionic and valeric acid) to PHV (Albuquerque et al. 2007; Lemos et al. 2006). With the given C-molar ratio of 45 ± 3 % to 55 ± 3 % of even to odd carbon atoms, a balanced ratio of PHB/PHV can be expected. This result can be used for a first indication which applications of the produced biopolymer are possible. Moreover, it is also important to ensure that the PHA compositions stays stable in order to meet market needs for stable product properties. With the obtained results over all season's primary sludge is a suitable raw material to ensure stable PHA-properties.



Volatile fatty acid	Mean ± Std. dev.	Min Max
Acetic acid	0.39 ± 0.03	0.34 0.45
Propionic acid	0.38 ± 0.04	0.31 0.45
Isobutyric acid	0.02 ± 0.01	0 0.03
Butyric acid	0.12 ± 0.01	0.10 0.14
Isovaleric acid	0.03 ± 0.01	0.02 0.04
Valeric acid	0.06 ± 0.01	0.04 0.09
Caproic acid	0.01 ± 0.01	0 0.04

Figure 5-1: VFA-composition of the acidified primary sludge batches (n = 31)

Like the VFA-composition all other parameters showed no seasonal dependency as well and are therefore summarized in Table 5-1 and not graphically displayed over the time. The average VFA-concentration was 7.5 g COD/L ± 2.3 g COD/L with a relatively high range of variation from 2.6 g COD/L (min) to 11.6 g COD/L (max) (see Table 5-1). Bengtsson et al. (2017) stated that the VFA-concentration should be at least higher as 2 g COD/L or rather in the range of 5-30 g COD/L for the PHA-accumulation, which was achieved with every batch. A suitable VFA-concentration for the biomass enrichment depends on the chosen organic loading rate (OLR) in combination with the hydraulic retention time (HRT). Literature shows that the range of applicable OLRs to successfully select PHA producing mixed microbial communities (MMC) fed with VFA

from waste streams is relatively wide. E.g. Valentino et al. (2020) used an OLR-range of 2.0-4.4 g COD/ (L d) with a HRT of 1 d, Dionisi et al. (2006) an OLR-range of 8.5–31.25 g COD/L with a HRT of 1 d and Campanari et al. (2014) an OLR-range of 4.7-8.4 g COD/ (L d) with a HRT of 1 d. Considering a strategy to minimize the necessary VFA-stream for the biomass enrichment, a lower OLR seems more suitable for a more efficient process. Thereby aeration costs for the biomass enrichment can be reduced and more VFA-solution is available for the PHA-accumulation to increase the overall PHA-product yield. The mentioned OLRs and HRTs from the literature would lead to a necessary VFA-concentration range of 2.0-31.25 g COD/L, which was also achieved with the acidified primary sludge.

Table 5-1: COD of input primary sludge ($COD_{tot,in}$) and composition of acidified primary sludge

Parameter	Unit	Mean ± Std. dev.	Min / Max	n
$COD_{tot, in}$	g/L	41.1 ± 8.3	22.8 / 58.2	28
$COD_{VFA, out}$	g/L	7.5 ± 2.3	2.6 / 11.6	31
VFA-yield ($COD_{VFA, out}$/ $COD_{tot, in}$)	-	0.19 ± 0.07	0.07 / 0.43	28
$COD_{sol, out}$/$COD_{tot, in}$	-	0.26 ± 0.09	0.13 / 0.58	26
$COD_{VFA, out}$/$COD_{tot, out}$	-	0.67 ± 0.10	0.37 / 0.83	27
$COD_{VFA, out}$/$COD_{sol, out}$	-	0.75 ± 0.09	0.55 / 0.90	26
$COD_{sol, out}$/$COD_{tot, out}$	-	0.89 ± 0.06	0.68 / 0.98	26
TKN	mg/L	508 ± 122	220 / 725	26
NH_4-N	mg/L	369 ± 81	138 / 465	24
P_{tot}	mg/L	149 ± 78	50 / 454	26
PO_4-P	mg/L	99 ± 34	39 / 162	23
COD_{VFA} : NH_4-N : PO_4-P	-	100 : 5.0 (± 1.2) : 1.3 (± 0.3)	-	22
COD_{sol} : NH_4-N : PO_4-P	-	100 : 3.7 (± 0.6) : 1.0 (± 0.2)	-	21
$COD_{tot, in}$: TKN : P_{tot}	-	100 : 4.4 (± 0.9) : 1.2 (± 0.4)	-	25

For a continuous biomass enrichment and PHA-accumulation, the observed rather high variation in the VFA-concentration leads to two process variations. The first one is using a constant volume flow of VFA and by that allowing a variation of the OLR during the biomass enrichment. However, usually is the OLR chosen constant which leads to the second variant. In this case a mixing and equalizing tank must be included after the solid/liquid-separation step to stabilize the VFA-concentration and it would be necessary to monitor the VFA-concentration, preferably online for a quick adaption of the volume flow. However, a full-scale plant could easily implement a continuous acidification as it is well-established for conventional anaerobic digestors. Assumingly this would lead to a more stable VFA-concentration within the acidification, high mixing and equalizing tank capacities might not be necessary. Because of the well documented success, producing PHA and therefore to be able to focus on other questions for closing the process chain, OLRs as constant as possible were chosen in this project by using an intermediate VFA-storage tank and adapting the volume flows. Nevertheless should the first variation also investigated in future to determine the allowable flexibility of the VFA-concentration of the VFA-feed during the biomass enrichment.

The VFA-yield was 0.19 ± 0.07 (see Table 5-1). It can be assumed that the yield could be increased if a more efficient heating system would have been installed. The heating jacket in combination with an unfavourable surface area to reaction volume ratio of the pilot scale reactor led to a relatively long time until the complete volume reached the desired temperature of 35 °C (up to 24-48 h). It is obvious that a high VFA-yield would result in higher PHA production capacities, but besides a high VFA-content, the feed for the following process steps should also meet requirements for the nutrient ratio (C:N:P). Depending on the

calculation method the C:N:P-ratio was in range for optimal aerobic biomass growth conditions (COD_{VFA}-based) resp. showed a little shortage of N (COD_{sol}- and COD_{tot}-based) as shown in Table 5-1. On the one hand, a continuously running biomass enrichment needs enough nutrients for biomass growth. It is not uncommon that nutrients are added into the VFA-feed to prevent growth limitation during the biomass enrichment as described in other studies (Tamis et al. 2018; Pittmann und Steinmetz 2014; Jia et al. 2014). For an up-scaling this would lead to additional process costs if external nutrients need to be dosed. A more cost efficient alternative could be to use internal nutrient rich streams from the STP, like turbid water from thickening or dewatering sludge streams if available. On the other hand, it is often documented that a nutrient limitation during the PHA-accumulation step induces an additional PHA-storage effect (Bengtsson et al. 2008; Montiel-Jarillo et al. 2017) and therefore a VFA-feed with nutrient limitation would be more desirable in this case. More convenient would be to use the produced VFA-feed without further adaptations. Morgan-Sagastume et al. (2014) showed first results that is possible to achieve a high PHA-content even without nutrient limitations using a VFA-feed produced from waste activated sludge. If it is also the case for a VFA-feed from primary sludge to realize high enough PHA-contents in the accumulation with a nutrient limitation as well as with a nutrient surplus, this would allow a wider process operation range for STP-operator and planer in the future. A VFA-feed without nutrient limitation could be used without the need for stripping ammonia or precipitating phosphorous. A VFA-feed with nutrient limitation could be used as well, if cheap possibilities exist to increase the nutrient content for the biomass enrichment which also allows strategies to uncouple the carbon from the nutrient feed like suggested in (Valentino et al. 2017). Another addition to these possibilities might be regulating the nutrient content during the acidification. Looking at Figure 5-2 indications can be observed that higher VFA-yields might lead to lower COD_{VFA}/NH_4-N-ratios and lower VFA-yields to higher COD_{VFA}/NH_4-N-ratios, whereas the COD_{VFA}/PO_4-P-ratio seems to be independent from the VFA-yield. If this proves true, an acidification process strategy could be designed not only to maximize the VFA-yield but also to aim for the desired nutrient-ratio. As the results were close to optimal conditions it was decided to use the VFA-feed as pure and unconditioned as possible.

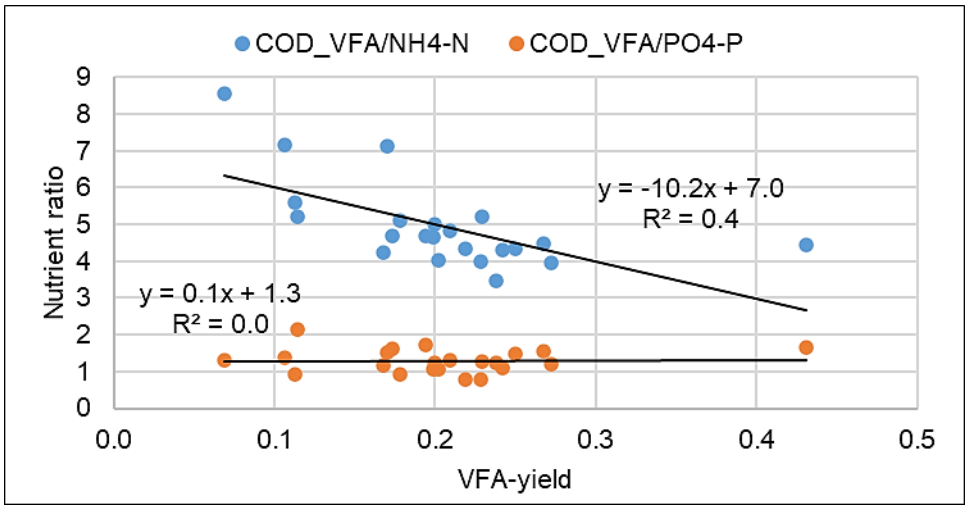


Figure 5-2: Nutrient-ratio in dependency of the VFA-yield

Another important aspect is the share of non-VFA-based COD after the acidification and solid/liquid-separation. In Table 5-1 it is listed that the share of COD_{VFA} on the COD_{tot} was only 0.67 ± 0.10 and the share of COD_{sol} on the COD_{tot} was 0.89 ± 0.06. This means that 33 % in the VFA-feed is non VFA-based COD (22 % soluble non-VFA COD and 11 % particular COD). In literature it is commonly pointed out that a high share of non-VFA based COD reduces the selective pressure to enrich MMC with high PHA production

capacities e.g. (Bengtsson et al. 2008). Technically the VFA-ratio could be increased by improving the solid/liquid-separation, either by adding conditioning agents to flocculate the fine particles for a better separation (Morgan-Sagastume et al. 2015) or by using an additional membrane filtration for particle separation (Valentino et al. 2020). However, both possibilities would lead to higher process and investment costs and finally in a higher product price, furthermore they would not affect the soluble non-VFA COD part. As it was one projective within this project to minimize as many aid flows and additional process steps as possible, no further adaption steps were foreseen to examine if a PHA-production is nevertheless possible.

5.2 PHA-production

pH was neither controlled in the biomass enrichment nor in the accumulation stage to reduce operational costs. Figure 5-3 shows typical pH-courses during the biomass enrichment and the PHA-accumulation. After a VFA-feed in the enrichment the pH dropped from ca. 8.0-8.1 to around 6.9 and steadily increased afterwards to a short peak of 8.2-8.3 and evened out to 8.0-8.1 again (see Figure 5-3 A). In all other runs the pH-value varied slightly but stayed in the same range. The PHA-accumulation was operated as fed-batch, so every 30 min a feed was added. In Figure 5-3 B it can be observed that with every feed the pH dropped slightly and increased after the VFA were consumed. Over the 24 h of the accumulation the pH gradually decreased from ca. 8.3 to 7.8 as the biomass slowly got saturated. The pH-drop at around 21:00 was because of a temporary aeration dropout. It is reported that a high PHA production rate can be expected if the reactors are operated in range of 7.5-8.5 (Villano et al. 2010). As the observed pH course is already in that range a pH control seems not necessary whereby the use of aid flows can be avoided to reduce operational costs.

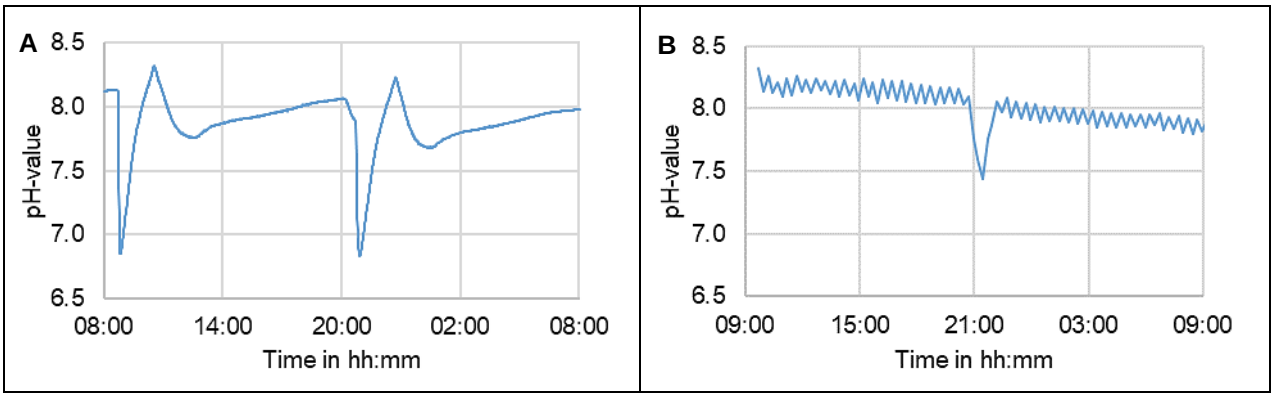


Figure 5-3: Typical pH-course during A: biomass enrichment and B: PHA accumulation

As mentioned in chapter 5.1 the OLR for the biomass enrichment ranges in literature in a wide range (~2-31.25 g COD/L). To reduce the VFA-consumption for the enrichment one set of experiments was done with a relatively low OLR (see Table 5-2). The first enrichment run was done for two weeks during wintertime with a temperature-range of 11.3-16.8 °C and two more runs were done during summertime for two resp. three weeks with temperature-ranges of 16.8-24.4 °C resp. 19.2-24.2 °C. The nutrient-ratios during three enrichment runs fluctuated depending on the calculation method around a light shortage resp. a sufficient nutrient supply. In the first run PO_4-P was not measured.

A first method commonly used in other studies to determine if the biomass enrichment was successful is to check the ratio of the feast-phase to a complete cycle (feast/cycle-ratio; F/C-ratio). If the F/C-ratio lies under the threshold of 0.2 described by Dionisi et al. (2007) it can be expected that the selection pressure during the biomass enrichment was sufficient. In all three enrichment runs with low OLRs F/C-ratios less or equal

to 0.2 resp. were observed, only in the case of the first week of the first run was the F/C-ratio with 0.23 slightly above 0.2 (see Table 5-2). On that basis an effective biomass enrichment could be expected.

Table 5-2: Parameters during biomass enrichment with low OLRs

Parameter		Unit	Run 1: 20.01.21-02.02.21	Run 2: 10.08.21-23.08.21	Run 3: 24.08.21-13.09.21
Enrichment					
OLR-range	COD_{tot}	g/(L d)	0.9-1.1	1.3-1.4	0.8
	COD_{sol}		0.8	1.1-1.2	0.6-0.7
	COD_{VFA}		0.7-0.8	0.8	0.5
COD_{VFA}/COD_{tot}		-	0.70	0.59-0.60	0.57-0.61
COD_{VFA}/COD_{sol}		-	0.83-0.90	0.67-0.75	0.70-0.84
Temperature	Mean	°C	13.8	22.2	21.5
	Min		11.3	16.8	19.2
	Max		16.8	24.4	24.2
COD_{tot} : NH_4-N : PO_4-P	1. week	-	100 : 2.7 : n.v.	100 : 3.6 : 1.1	100 : 2.4 : 0.7
	2. week		100 : 3.2 : n.v.	100 : 2.6 : 0.6	100 : 2.9 : 0.7
	3. week		-	-	100 : 3.4 : 0.7
COD_{sol} : NH_4-N : PO_4-P	1. week	-	100 : 3.2 : n.v.	100 : 4.0 : 1.3	100 : 2.8 : 0.8
	2. week		100 : 3.6 : n.v.	100 : 3.3 : 0.8	100 : 3.4 : 0.8
	3. week		-	-	100 : 4.9 : 1.0
COD_{VFA} : NH_4-N : PO_4-P	1. week	-	100 : 3.5 : n.v.	100 : 6.0 : 1.9	100 : 3.9 : 1.1
	2. week		100 : 4.4 : n.v.	100 : 4.4 : 1.1	100 : 4.9 : 1.1
	3. week		-	-	100 : 5.9 : 1.2
Feast/cycle-ratio (mean of 2 SRTs prior to accumulation)	1. week	-	0.23 ± 0.03	0.14 ± 0.02	0.17 ± 0.04
	2. week		0.17 ± 0.01	0.20 ± 0.04	0.14 ± 0.02
	3. week		-	-	0.12 ± 0.03
Accumulation					
Mean temperature	1. week	°C	13.1	20.7	23.0
	2. week		14.5	23.0	23.2
	3. week		-	-	22.9
PHA/VSS wt. ratio (HB/HV-molar ratio)	1. week	%	49 (41/59)	32 (48/52)	29 (46/54)
	2. week		54 (42/58)	27 (49/51)	26 (45/55)
	3. week		-	-	21 (43/57)

As shown in Table 5-2 the highest PHA-contents referred to VSS were achieved in the first run (during wintertime) with 49 % after the first week of enrichment and 54 % after the second week. These results confirm that a PHA production is possible during German wintertime without heating systems for enrichment or accumulation reactors.

During the second and third run the PHA-contents were lower which might be led back to the VFA-share of the COD_{tot}. In both runs the COD_{VFA}/COD_{tot}-ratios with 0.59-0.60 and 0.57-0.61 were distinctly lower compared to the first run with 0.70 which could have favored a development of a side population that did not produce PHA during the biomass enrichment and accumulation as described by (Morgan-Sagastume et al. 2015; Valentino et al. 2018). For planers and operators it is important to know the threshold to aim for when designing and operating PHA-production facility fed with waste/wastewater-streams. Valentino et al.

(2020) used the organic fraction of municipal solid waste (OFMSW) and OFMSW mixed with waste activated sludge (WAS) for producing a VFA-feedstock for the biomass enrichment. Solids were completely removed by centrifugation and an additional ceramic membrane filtration. With the resulting COD_{VFA}/COD_{sol}-ratios of 0.85 (OFMSW) and 0.73 (OFMSW-WAS) MMCs were cultivated which were able to accumulate PHA in a range of 36-48 % (PHA/VSS) with OFMSW and 39-44 % with OFMSW-WAS. Tamis et al. (2018) were able to enrich a biomass which achieved PHA-contents of up to 70-80 % (PHA/VSS) with a fermented solid free wastewater from a paper mill with a COD_{VFA}/COD_{sol}-ratio of 78 % under well optimized accumulation environment and already 50 % (PHA/VSS) after the feast-phase of the enrichment. Amulya et al. (2015) cultivated a biomass with fermented food waste (COD_{VFA}/COD_{sol}-ratio of 36 %) which was able to accumulate only 24 % (PHA/CDW) (which correlates to 27-34 % (PHA/VSS) with an assumed organic content of 70-90 %). Jia et al. (2014) reached a PHA-content of up to 59 % (PHA/CDW) by a biomass fed with fermented excess sludge containing 65 % COD_{VFA} of the COD_{tot}. It is obvious that the final PHA-content depends on many parameters but summarizing the mentioned results a threshold could be derived that PHA-contents of approx. 35 % (PHA/VSS) are unlikely to be exceeded when the COD_{VFA}-content in the VFA-feed is under approx. 65 %. It was not the goal to optimize the acidification or the subsequent solid/liquid-separation during this project but nevertheless, even with little effort the COD_{VFA}/COD_{tot}-ratio in 16 out of 27 acidification batches was higher and 7 additional batches would have met the requirement if all solids were removed. For planning up-scaled plants this means that high PHA-contents are favored by high VFA-shares but it is not necessary to maximize it, if the requirements are already fulfilled with little technical effort which can save investment and operational costs.

The HB/HV-ratios (see Table 5-2) were relatively stable in all accumulation batches (41-49 % HB to 51-59 %) which is due to the stable VFA-composition shown in Figure 5-1 (see chapter 5.1). The average composition of VFAs with a number of even to odd carbon atoms was 45 ± 3 % to 55 ± 3 % (C-molar-basis), which perfectly correlated to the HB/HV-ratio. Considering also the accumulation batches which are described in the following the HB/HV-ratio was 47 ± 7 % to 53 ± 7 % (n = 14) and thereby also in the expected range.

One problem during the operation of these three enrichment runs was the low total suspended solid content (TSS). Without a considerable TSS a high PHA-content alone seems not sufficient as the overall product yield and the technical effort is no longer in proportion. After the inoculation of the enrichment reactor the TSS was in the range 4.4-7.8 g/L and dropped to 0.6-0.8 g/L in all runs after two weeks and even further to 0.5 g/L after three weeks in run three. In accordance to the worksheet (DWA A-131, 2016) the applied OLR, SLR and HRT was adapted in order to increase the surplus sludge production and thereby be able to aim for a TSS of 2 g/L. The OLR was set to 2.7-2.9 g COD_{tot}/(L d) (2.1 -2.3 g COD_{VFA}/(L d)), SLR and HRT to 2 d resp. 1 d.

It was observed that the higher OLR could not increase the solid content. The TSS dropped from 4.1 g/L to 0.2 g/L after 4 weeks of operation. After the first and second week of enrichment the PHA-content was only 0.02 resp. 0.08 g PHA/g VSS and reached only 0.16 g PHA/g VSS after four weeks. The higher OLR without adapting the overall cycle length led to a feast/cycle-ratio of 0.86 ± 0.13 which did not decrease over the course of the enrichment run, thus resulting in an insufficient selection pressure for PHA-producing bacteria. The resulting higher feed/mass-ratio compared to the trials with lower OLRs led most probably to the high feast/cycle-ratio. A possible explanation for the low TSS could be, among other things, that nutrient supply was not high enough for biomass growth, however this stands in contrast to Valentino et al. (2020) who were able to maintain a VSS of 1.0-1.4 g/L (roughly corresponds to a TSS of 1.4-2.0 g/L) with a similar OLR-range of 2.0-3-3 g COD/(L d) and a more pronounced nutrient limitation (100 : 3.6 : 0.8; COD_{VFA} : NH_4-N : PO_4-P (g)). It cannot be excluded that the low solid content might have also been caused by technical

limitations of the used reactors. It is possible that the submerged pumps withdrew solids after the sedimentation even with an installed pump chamber in the reactor.

To reduce sludge loss, a process variation was tested in which no withdrawal from the mixed liquor was done, for this a sedimentation phase was implemented within every cycle. The OLR was in range of 2.1-2.6 COD_{tot}/(L d) (1.5 -1.8 g COD_{VFA}/(L d)) (n = 4) and 1.1 COD_{tot}/(L d) (0.5 g COD_{VFA}/(L d)) (n = 1). The SRT was 6 d and the HRT 2 d. With this approach a TSS of 2.9-4.7 g/L was achieved by the end of the enrichment but probably because of the high share of non PHA-producing bacteria the PHA-content was only 10-14 % (PHA/VSS) (n = 5).

5.3 Biological composition

Biological samples from 8 of the acidification batches as well as from the VFA-storage tank were taken to investigate the stability of the composition of the anaerobic microbial community. Furthermore, samples from the enrichment experiments were taken regularly to examine the effect of the enrichment on PHA-producing organisms. The 40 most abundant genera over all samples were selected and are shown in the following figures. The remaining genera were summarized and are referred to as “other prokaryotes”.

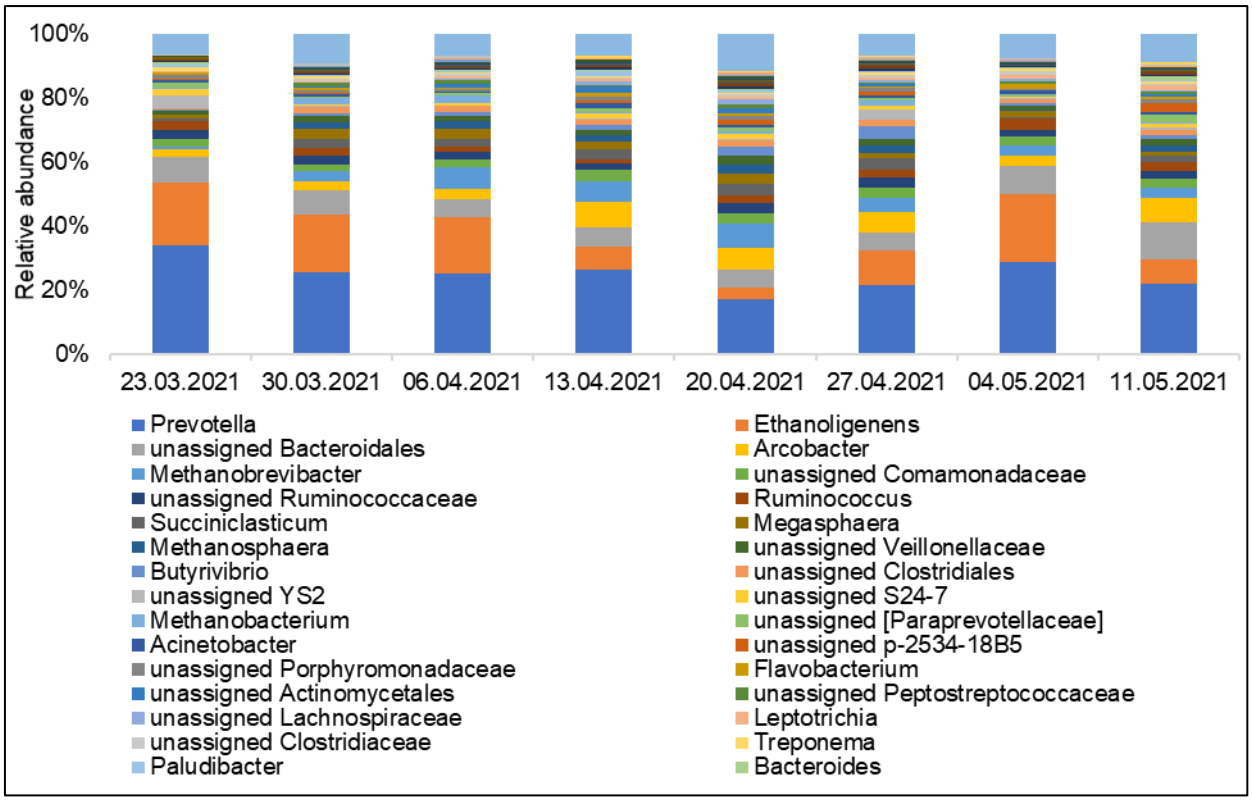


Figure 5-4: Microbial composition at the end of acidification batches

Figure 5-4 shows the biological composition of the acidified primary sludge at the end of the acidification batches. The most dominant genera were *Prevotella* with a mean relative abundance of 24.9 ± 4 6 % and *Ethanoligenes* with a mean relative abundance 13.2 ± 6.4 % over all samples. Furthermore, *Arcobacter* (5.1 ± 2.1 %) and an unassigned member of the order Bacteroidales (7.3 ± 1.9 %) were abundant organisms within the samples. These taxa are known for their ability to produce VFAs under anaerobic

conditions (Lin et al. 2016; Li et al. 2019; Xin et al. 2021; Miceli et al. 2016). *Methanobrevibacter*, which is a hydrogenotrophic methanogen (Siegert et al. 2014) was another relatively abundant organism in most of the samples (4.4 ± 2.2 %). Overall, the biological composition within the acidification batches kept relatively constant with some exceptions, mainly on April 13th and 20th. This investigation fits well with the stable composition of the produced VFAs explained in chapter 5.1. But the slight shifts in the population abundances may explain the varying VFA-concentration produced, especially variations in the abundances of VFA-producing and methane-producing organisms.

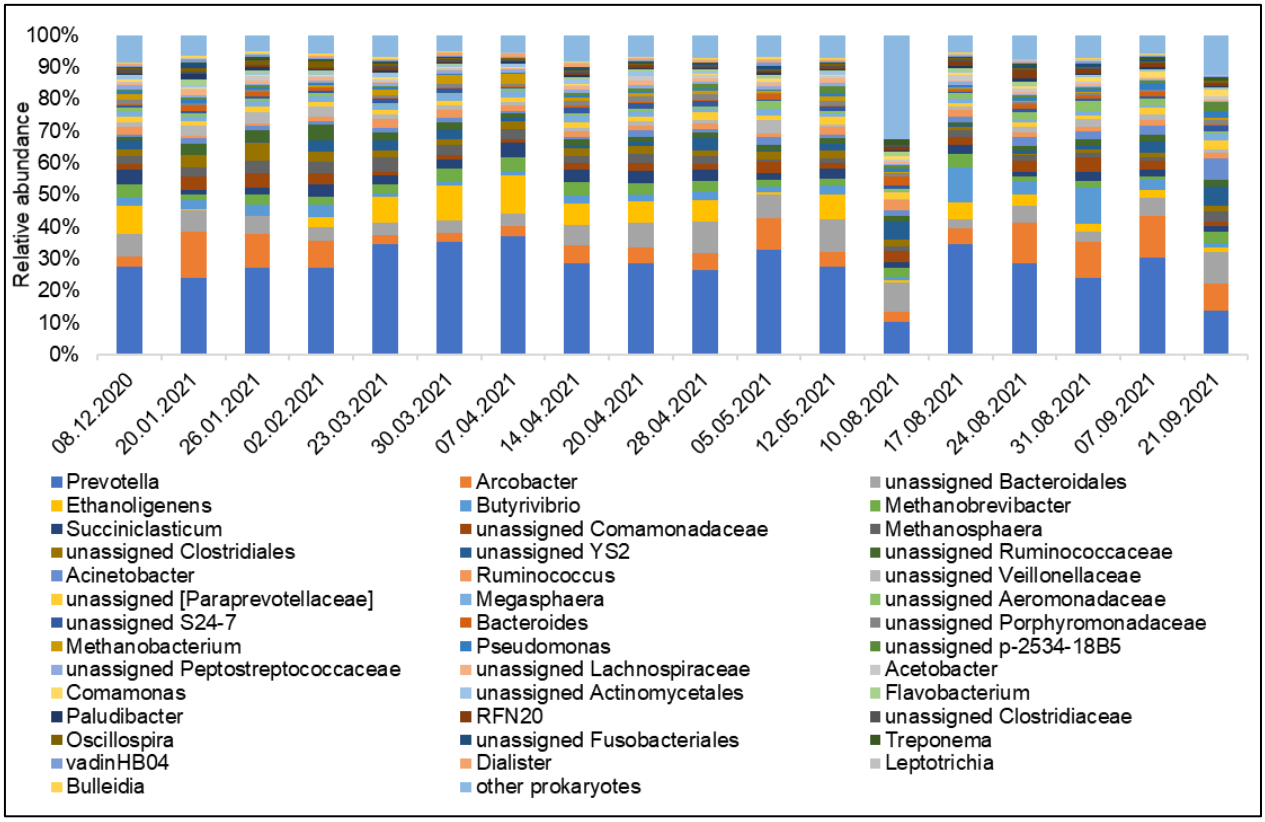


Figure 5-5: Microbial composition of the VFA-storage tank

The most dominant taxa in the acidified primary sludge *Prevotella* (27.6 ± 6.7 %), *Arcobacter* (7.1 ± 3.8 %), a member of the Bacteroidales (6.4 ± 2.3 %) and *Ethanoligenes* (4.9 ± 3.6 %) were also the most abundant organisms in the VFA-storage tank (Figure 5-5). When comparing Figure 5-4 and Figure 5-5 it should be noted that after each acidification batch the VFA-feed was due to working routines mostly temporarily stored for 1 d before it was pumped into the VFA-storage tank. *Methanobrevibacter* showed a slightly lower relative abundance in the storage tank samples compared to the acidification batches (3.0 ± 1.1 %). Although organisms that are capable of conversion of carbon compounds into volatile fatty acids under anaerobic conditions were present in the storage tank, however the VFA-concentration remained stable in the tank which was mentioned in chapter 4.3. This may be caused by the low temperature in the storage tank, which was kept at ambient temperature in contrast to the acidification reactor which was run at a temperature of 37 °C. This fact may have prevented the dominant fermenting bacteria in this system from converting more of the available COD into VFAs. Another possible reason for this may be that the remaining COD was an inert fraction which could not be further processed by the microorganisms. In conclusion the storage of the

substrate in the VFA tank resulted in a relatively stable microbial composition as well as a stable VFA-composition and -concentration.

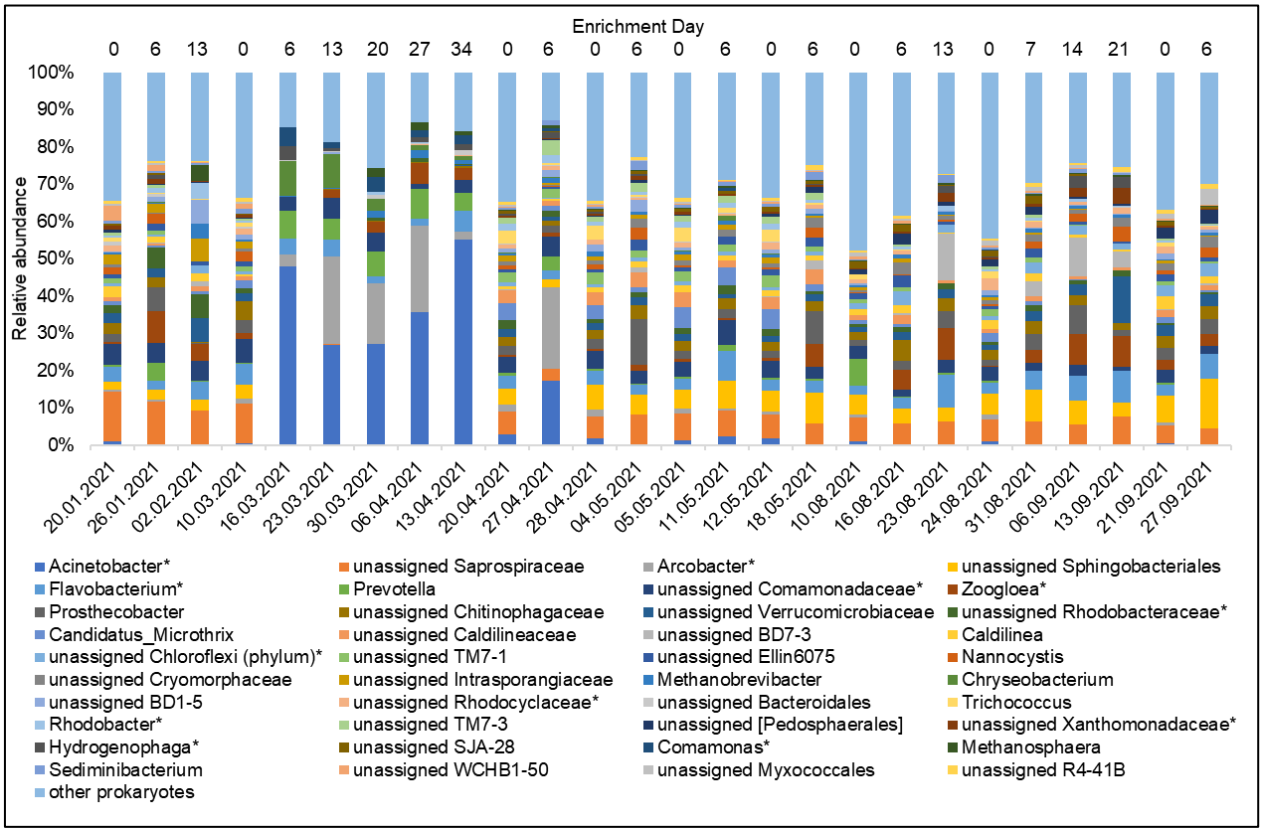


Figure 5-6: Microbial composition within the enrichment experiments. Stars indicate taxa with the potential ability to produce PHA

The microbial community of the activated sludge used for the enrichment experiments on day 0 showed a comparable composition (Figure 5-6) with members of the Saprospiraceae, Sphingobacteriales and Comamonadaceae and the genus *Flavobacterium* being the most abundant taxa in these samples with mean relative abundances of 7.4 ± 2.6 %, 5 ± 1.4 %, 4.5 ± 0.9 % and 3.5 ± 0.8 %. Clear shifts in the community composition after several weeks of enrichment could be observed in most of the experiments. The most dominant taxa with the ability of producing PHA over all samples were Acinetobacter (8.1 ± 15.1 %), Arcobacter (4.2 ± 7.4 %), Flavobacterium (4.0 ± 2.1 %), a member of the Comamonadaceae (3.8 ± 1.4 %) and Zooglea (2.8 ± 2.8 %). In the experiments with the highest OLR (10.03.21-13.04.21 and 20.04.21-27.04.21) Acinetobacter and Arcobacter showed a high increase in their abundances from 0.6 to 55.2% and 2.9 to 17.4% (Acinetobacter) and 1.3 to 2.2 % as well as 1.7 to 21.9 % (Arcobacter), respectively. In the remaining experiments the potential PHA producers which showed the highest increases in their abundance over time were mainly *Zooglea*, *Flavobacterium* (0.9 ± 0.8 % to 4.1 ± 3.0 %; 3.4 ± 0.9 % to 5.8 ± 2.5 %). This may lead to the assumption that different organisms with the potential to produce PHA may proliferate under different environmental parameters. Interestingly, some of the organisms which had an increasing relative abundance in some of the enrichment experiments were organisms that were also relatively abundant in the substrate and had a low abundance in the starting community of the activated sludge. These were for example Arcobacter and Prevotella, which were dominant organisms in the substrate (7.1 ± 3.8 % and 27.6 ± 6.7 %) but rare organisms in the activated sludge (1.2 ± 0.4 % and 1.3 ± 2.1 %). An

influence of the bacteria inserted by the feed on the PHA community, either positive or negative, cannot be excluded and should be investigated in more detail. With this, statements concerning the necessary solid-liquid-separation for the operator could be made. Furthermore, a more detailed investigation of the involved microorganism in the different process steps could help for a better planning of the process or to have an idea of the resulting VFA- as well as PHA-yield and composition.

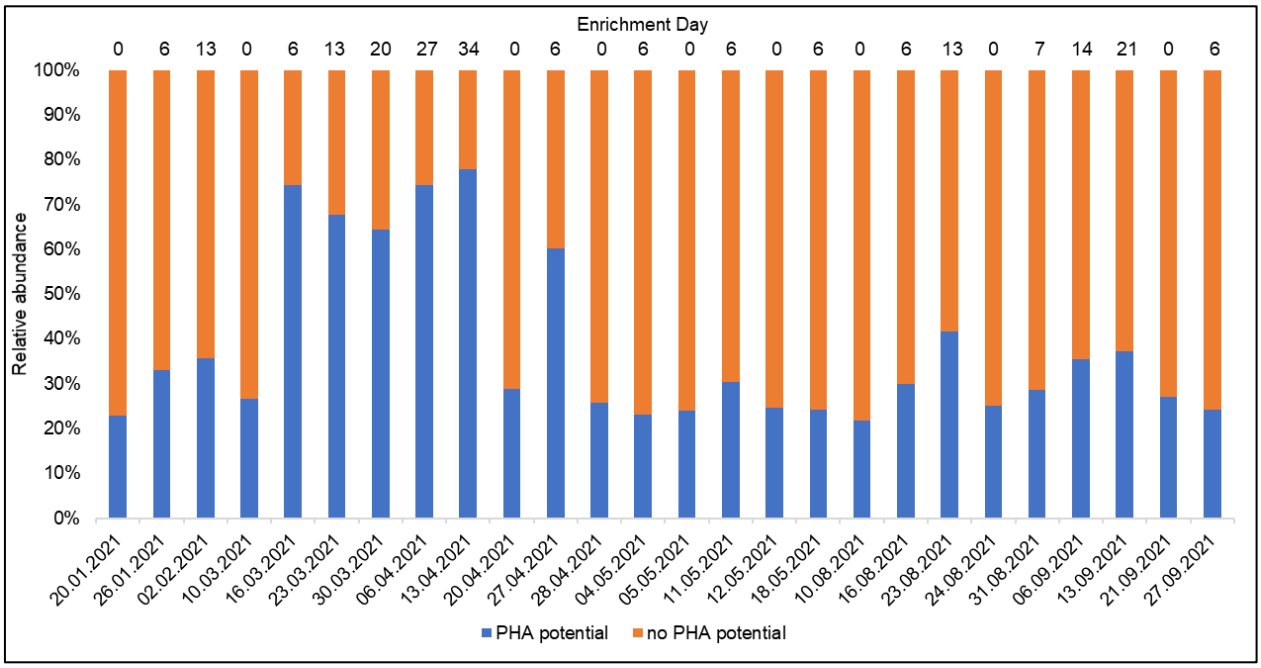


Figure 5-7: Relative abundance of potential and non-potential PHA producers during the enrichment experiments

Figure 5-7 shows the cumulative relative abundance of the microorganisms with a potential to produce PHA and organisms without this trait over the enrichment experiments. An increasing abundance of potential PHA producers with each week of enrichment could be observed in most of the experiments except for three of the 6-day batch experiments conducted from 28.04.-04.05.21, 12.05.-18.05.21 and 21.09.-27.09.21. In fact, in these three experiments the abundance of potential PHA producers slightly decreased from 25.6 to 23.1 %, from 24.6 % to 24.3 % and from 27.1 % to 24.3 %. The abundance of potential PHA producing organisms was only slightly increasing in the remaining 6-day experiment (05.05.-11.05.21) from 23.9 to 30.4 %. This finding is in line with the low PHA content produced in these experiments (10-14 % PHA/VSS; see chapter 5.2).

The experimental runs conducted from 10.03.-13.04.21 and 20.04.-27.04.21 showed the highest increases in the relative abundance of potential PHA producers from 26.5 % at day 0 to 77.9 % at day 34 and from 28.8 on day 0 to 60.3 % on day 6. However, in these experiments only low amounts of PHA with 15 % and 9 % (PHA/VSS) were produced. Furthermore, the dominant potential PHA producing taxa in these runs differed from the most abundant possible PHA producers in the other experiments (see Figure 5-6). These two experiments were the ones with the highest applied OLRs (about 2 g COD_{VFA}/(L d)) and the substrate used in these runs also exhibited high COD_{VFA}/COD_{sol}-ratios of 0.77-0.89 and 0.80-0.86. Because of possible favorable growth conditions for the prevailing microorganisms with the potential for PHA production these substrate characteristics may have caused this observation. The low PHA amount on the other hand could be a result of the very short famine phase (see chapter 5.2) and a substrate saturation in the enrichment reactor which may have prevented the induction of the PHA production metabolism

(Nguyenhuynh et al. 2021) and in addition of a possible introduction of unconsumed substrate in the accumulation reactor.

A replacement of the enrichment with a short acclimation phase was therefore unfavorable in the pilot operation under these conditions. But, by applying a high OLR this problem may be overcome in future research approaches.

The best performing runs regarding PHA-contents produced were the experiments from 20.01.-02.02.2, 10.08.-23.08.21 and 24.08.-13.09.21 (21-51 % PHA/VSS; see chapter 5.2) although the relative abundance of potential PHA producers showed a lower increase compared to the two previous explained experiments (23 to 35.7 %, 21.8 to 41.6 % and 25.1-37.1 %). This may be caused by a lower feast/cycle-ratio which would induce a metabolism to produce PHA (Nguyenhuynh et al. 2021) because energy reserves are needed during the longer phases without substrate availability. Though, the successful enrichment of potential PHA producers with an accompanying improvement in PHA production may result from the high ratios of COD_{VFA} to COD_{sol} (see chapter 5.2).

In conclusion the produced PHA-amount can not only be predicted by the abundance of potential PHA producers, but also other factors for example an appropriate substrate concentration and feast-to-famine-ratio to induce the PHA production metabolism should be considered. The growth of PHA producers on the other hand can be improved by knowing the key organisms and changing the parameters to their optimal growth conditions. The knowledge and monitoring of the microbiological sludge composition may be advantageous for operators to adjust parameters for the needs of PHA producers to optimize the PHA amount that can be produced.

5.4 PHA-stability after accumulation

To determine a possible PHA-loss of unstabilized PHA-enriched biomass after the accumulation, various degradation tests were done. The first tests were conducted directly after the PHA-accumulation to simulate the time until thickening and dewatering can be finished. The second set of tests were done with sludge after dewatering to see the influence of the drying time (cf. chapter 4.5).

In Figure 5-8 the PHA-loss is shown for untreated samples taken directly after the accumulation or after dewatering and of VFA-stabilized samples after the accumulation. The PHA-loss in the following was calculated based on percentage points (e.g. decrease from 30 % to 23 % means loss of 7 %).
The PHA-content of the untreated samples taken after the accumulation was stable for 1 h and started to decrease afterwards. Until 6 h after sampling, the loss reached 7.3 % ± 0.3 % from its initial content. The dewatering step showed no influence on the PHA-stability in the centrifuged sludge. The PHA-content gradually decreased by 4.3 % after 6 h. Both results indicate that the accumulated PHA is not or only for a short period of time stable in the cells, which is probably not long enough for a possible sedimentation phase for thickening plus the time for dewatering larger volumes considering full scale reaction volume size and the needed time until the biomass is completely dried. To examine if the PHA-content can be stabilized without adding chemicals or rather only by using internal process flows an additional degradation test was done with samples taken directly after the accumulation but stabilized with VFA produced from acidified primary sludge to a pH-value of ca. 5.5. This was achieved with a mixture of ¼ of VFA (pH ca. 4.9-5.0) and ¾ of sample volume (pH ca. 7.5-8.0). Figure 5-8 shows that the PHA-content of the VFA-stabilized samples stayed relatively constant over the full observation period of 6 h (0.0 % ± 0.3 %). With this method an additional usage of the VFA-feed would be necessary but if the VFA-concentration of the liquid phase after dewatering is still high enough with the chosen mixing ratio it could be recycled within the PHA-process.

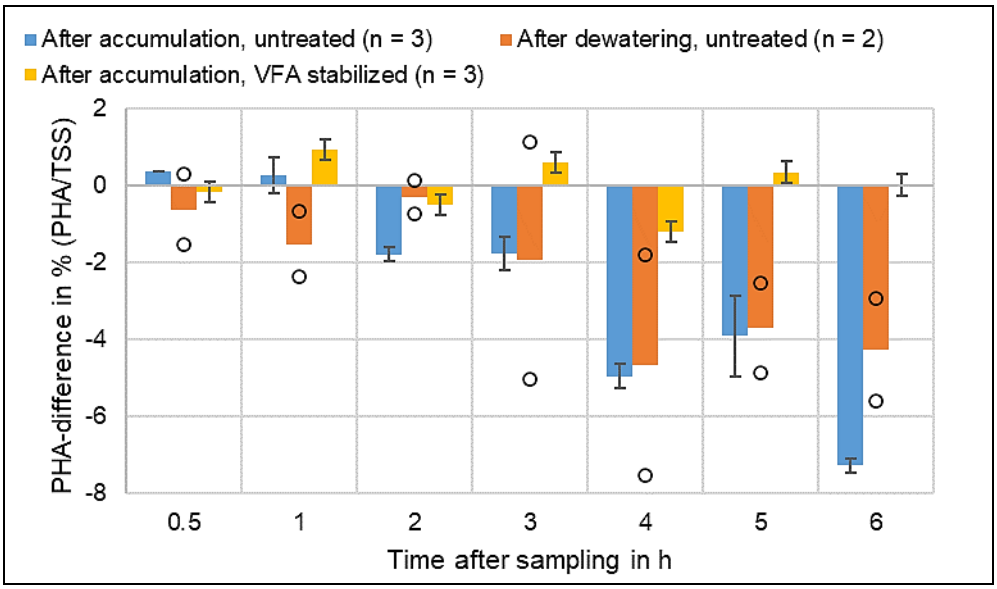


Figure 5-8: Average PHA-stability over 6 h after the accumulation and after dewatering (PHA-difference displayed in percentage points)

For further evaluations of a PHA-degradation caused by the necessary processing time after the accumulation and by the drying step, in Figure 5-9 PHA-contents of sludge samples are compared, which have been taken after different process steps and differently handled. In Figure 5-9 A the legend describes after which step the samples were taken and how they were treated afterwards. By comparing the different sample types, it is possible to determine what effect the whole down-streaming process and only the drying have on the PHA loss (see legend Figure 5-9 B). As described in Figure 5-9 B the drying step could have two different effects which cause a PHA-loss, the necessary time for drying and the unfavourable extraction surface after drying.

It should be noted that the PHA-content in Figure 5-9 is shown based on VSS as the adding of sulfuric acid and the washing step influence the ratio of the inorganic/organic matter.

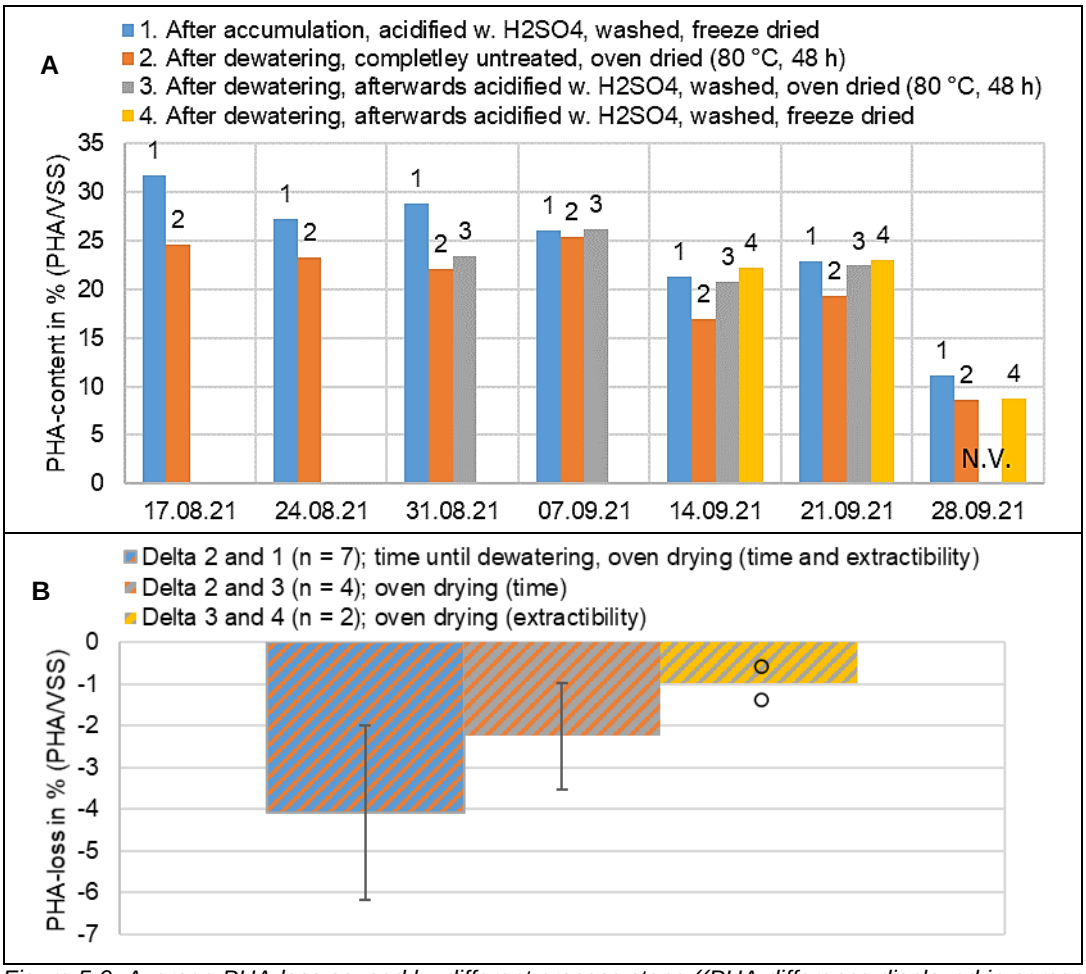


Figure 5-9: Average PHA-loss caused by different process steps ((PHA-difference displayed in percentage points)

The comparison between columns with number 1 and number 2 in Figure 5-9 A is shown in Figure 5-9 B as the first column. It can be observed that the untreated, oven dried PHA-enriched biomass lost 4.1 %± 2.1 % PHA compared of its initial content, what emphasises again the necessity of a stabilization step or rather a short time until the biomass is dried to avoid losses. The loss caused by the time until the sludge was dried in the oven (difference of columns 2 and 3 in Figure 5-9 A, second column in Figure 5-9 B) accounted for 2.3 %± 1.3 %. However the drying time was not the only reason for the loss, it seems that the extractability (difference of columns 3 and 4 from Figure 5-9 A, third column in Figure 5-9 B) contributed a small loss of 1 % of PHA. Both effects should be minimized by choosing a drying procedure which can dry biomass relatively fast if left untreated and creates a powdery dried sludge with a high surface.

This leads to the conclusion that stabilization can be necessary if the dewatering and drying procedure after an accumulation are expected to last several hours especially when the PHA-content is on the threshold for a feasible extraction process. By adding the internal process flow of VFAs from the acidified primary sludge no external chemicals are needed which can save money for chemicals and additional process equipment and favours a more sustainable process development and the liquid part of the solid/liquid-separation could be reused in the biomass enrichment or the accumulation step.

5.5 Trace-pollutions

As mentioned in chapter 4.4 it was tried to gain a rough first impression if trace pollutions might remain in the PHA enriched biomass and therefore be passed on through the process chain. Table 5-3 summarizes the loads which went into the accumulation and the loads which were in the liquid phase after dewatering of the PHA enriched biomass. For Carbamazepin, Diclofenac, Metoprolol and 4 resp. 5-Methyl-1H-Benzotriazol the load in the centrate was either even or higher compared to the load which went into the accumulation. The higher load after dewatering could be caused by an inaccuracy during sampling or the need for a simplified calculation method (see chapter 4.4). Nevertheless, these results give an indication that these substances did not remain or accumulate in the sludge. The only substance which showed a higher load in the inflow compared to the centrate was 1H-Benzotriazol. The displayed differences of 11904 µg to 3501 µg resp. 10417 µg to 1923 µg seem significant enough to expect that there might have been an accumulation in the PHA enriched sludge.

Table 5-3: Trace pollutions in process streams of the PHA accumulation

Trace pollutions	**Carbamazepin**	**Diclofenac**	**Metoprolol**	**1H-Benzotriazol**	**4+5-Methyl-1H-Benzotriazol**
in	µg	µg	µg	µg	µg
Accumulation batch: 21.09.21					
Load into accumulation	2230	353	1942	11904	3903
Load in centrate	3284	590	3583	3501	3657
Accumulation batch: 28.09.21					
Load into accumulation	3427	363	5077	10417	5456
Load in centrate	3846	366	3662	1923	7508

These results should only be considered as a very cautious assessment for a first impression. Furthermore, thorough investigations are needed to determine the fate of the trace pollutions. Those analyses should preferably be done in the solid streams.

6 Summary

Sewage contains valuable substances that can be used as raw materials for biobased products. However, to date this potential has hardly been exploited to its full potential in North-West Europe. It is important to lower technical obstacles for implementing technologies to recover carbon-based elements by demonstrating the feasibility and creating a knowledgebase for a practical implementation beyond lab scale studies. For this purpose, a pilot plant was operated to produce Polyhydroxyalkanoates, a biodegradable biopolymer solely from material flows of a municipal sewage treatment plant. It was important to examine if stable product properties can be reached while operating the pilot under all seasonal conditions without adding aid flows or chemicals to ensure a sustainable production process and to minimize operational costs.

For the first step within the whole PHA production chain a VFA-rich stream was generated by anaerobically acidifying primary sludge. It was observed that the VFA-composition was independent from any seasonal influences. The average VFA-ratio between VFAs with a number of even to odd carbon atoms was 45 ± 3 % to 55 ± 3 % (C-molar-basis). As this ratio indicates the PHA composition and thereby the properties these results suggest that primary sludge is assumingly a suitable raw material to ensure stable PHA-properties during all seasons. This was confirmed with the conducted PHA-accumulations runs which delivered a HB/HV-ratio of 47 ± 7 % to 53 ± 7 %.

The average VFA-concentration was 7.5 g COD/L ± 2.3 g COD/L which was in a suitable range for producing PHA. The rather high variation of the concentration was not caused by any seasonal influences. The variation suggests, when using primary sludge as a substrate, that either a fluctuating OLR during the biomass enrichment has to be allowed, or, if the OLR has to be kept constant, a combination of a mixing and equalizing tank with a preferably online monitoring of the VFA-concentration has to be implemented to adapt the volume flows into the following process steps.

The VFA-yield ($COD_{VFA,\ out}$/ $COD_{tot,\ in}$) was comparatively low with 0.19 ± 0.07. It is expected that a better heating system could improve the acidification. However, first results indicated that a partial acidification led to a higher COD_{VFA}/NH_4-N-ratio. Using this effect not only a high conversion of COD into VFA can be aimed for but also a desired nutrient ratio. By doing so an additional nutrient source for biomass growth during the biomass enrichment would not be necessary and could lower the process costs. The COD_{VFA} : NH_4-N : PO_4-P-ratio was 100 : 5.0 (± 1.2) : 1.3 (± 0.3) (or COD_{sol} : NH_4-N : PO_4-P 100 : 3.7 (± 0.6) : 1.0 (± 0.2)) and thereby in range of optimal conditions for biomass growth with a tendency of nitrogen shortage when including the non-VFA solvent COD into calculation.

Three biomass enrichment runs were performed in the OLR-range of 0.5-0.8 COD_{VFA}/(L d) (0.8-1.4 COD_{tot}/(L d)). The first run was during wintertime and the other two runs during summertime. Weekly accumulation batches resulted in PHA-contents (PHA/VSS) of 49-51 % (run 1), 27-31 % (run 2), 21-29 % (run 3). Thereby it was confirmed that a successful PHA-production process can be operated even without heating of the reactors. The difference in PHA-content of the three runs was presumably caused by the efficiency of the biomass enrichment. It is reported that a high COD_{VFA}/COD-ratio favours an efficient selection pressure to enrich PHA-producing bacteria. The overall observed COD_{VFA}/COD_{tot}-ratio was 0.67 ± 0.10 (with a COD_{sol}/COD_{tot}-ratio of 0.89 ± 0.06). In the first run it was 0.70 and in the other two runs 0.57-0.61. Reviewing literature and using these results a limit could be approximated. It appears that a COD_{VFA}/COD_{tot}-ratio should be at least over 0.65 to ensure a successful biomass enrichment process. Further investigations are necessary to prove this limit but if it proves true, operators and planers could use it for designing and operating the solid/liquid-separation. If it is already possible with little effort (e.g.

centrifugation) to achieve this threshold a sophisticated solid/liquid-separation using additional flocculants or membranes is not necessary.

One problem which was unmanageable during this project was the low TSS-content during the biomass enrichment. Additional enrichment runs were performed with a higher OLR (2.7-2.9 g COD_{tot}/(L d); 2.1 - 2.3 g COD_{VFA}/(L d)) and without biomass withdrawal. In the first case the biomass growth could not be increased, and the PHA-content (PHA/VSS) reached only 16 % after 4 weeks of biomass enrichment, assumingly due to an insufficient selection pressure indicated by a feast/cycle-ratio of 0.86 ± 0.13. In the second case the TSS was higher as no biomass was withdrawn but the PHA-contents (PHA/VSS) were only in range of 10-14 % and therefore considered not interesting for an extraction process.

The analysis of the microbial community of the acidified primary sludge showed a high relative abundance of VFA-producing organisms and a relatively stable composition. This finding fits well with the stable VFA composition produced during the acidification batches. The most abundant bacteria in the acidified primary sludge were found to be most abundant in the VFA-storage tank as well. Though, no further conversion of COD into VFAs could be observed which resulted in a stable substrate for the enrichment and accumulation experiments. In conclusion, the storage of the substrate in the VFA tank resulted in a relatively stable microbial composition as well as a stable VFA-composition and -concentration. In the experiments with the highest OLR (10.03.21-13.04.21 and 20.04.21-27.04.21) other potential PHA producers showed a high increase in their abundances from 0.6 to 55.2 % and 2.9 to 17.4 % (*Acinetobacter*) and 1.3 to 2.2 % as well as 1.7 % to 21.9 % (*Arcobacter*) compared to the remaining experiments where mainly *Zooglea*, *Flavobacterium* (0.9 ± 0.8 % to 4.1 ± 3.0 %; 3.4 ± 0.9% to 5.8 ± 2.5 %) were the most dominant potential PHA producers. This indicates that different organisms with the potential to produce PHA may proliferate under different applied parameters in the enrichment. Furthermore, the two experiments with the high OLR showed the highest increases in the relative abundance of potential PHA producers from 26.5 % at day 0 to 77.9 % at day 34 and from 28.8 % on day 0 to 60.3 % on day 6. However, in these experiments only low amounts of PHA with 15 % and 9 % (PHA/VSS) were produced. The low PHA amount could be a result of the very short famine phase (see chapter 5.2) and a substrate saturation in the enrichment reactor which may have prevented the induction of the PHA production metabolism. The three main runs with the best PHA amount produced (21-51 % PHA/VSS) showed an increase in potential PHA producers over time which was not as pronounced as in the previous mentioned two experiments (23 to 35.7 %, 21.8 to 41.6 % and 25.1-37.1 %). The better PHA production may be caused by lower feast/cycle-ratios, which could induce the metabolism for PHA production during the famine phase. In conclusion the produced PHA-amount can not only be predicted by the abundance of potential PHA producers, but also other factors for example an appropriate substrate concentration and feast-to-famine-ratio to induce the PHA production metabolism should be considered. Overall, a successful enrichment of potential PHA producers could be performed during the enrichment experiments, except for three of the four 6-day experiments. This aspect may be overcome by applying a higher OLR which was indicated by the remaining 6-day experiment.

The drying and dewatering after a PHA-accumulation could take several hours. It was observed that in the untreated mixed liquor the PHA-content was stable for 1 h and reached a loss of up to 7.3 % ± 0.3 % after 6 h. In the centrifuged untreated mixed liquor, the PHA gradually decreased by 4.3 % after 6 h. To avoid using external chemicals like H_2SO_4 or a high energy consumption for a thermal shock to stabilize the PHA-content during down streaming, the VFA-feed produced from the primary sludge was used to lower the pH to approx. 5.5 and thereby be able to stabilize the PHA-content in the mixed liquor up to 6 h only using internal process flows.

The operation in pilot-scale pointed out weak points in the process chain and made it possible to develop strategies for up-scaling. It could be shown that during an all seasons of the PHA operation a stable production process and therefore stable PHA product properties can be expected. The technical feasibility of producing PHA solely with streams from a municipal sewage treatment plant without addition of external aid flows seems to be possible. With further modifications for the PHA production and following steps of the process chain it should be possible to achieve the PHA amounts mentioned in the market potential report of the WOW! Project (WIW 2020) and thereby contributing to reduce the production of conventical non-biodegradable polymers from crude oil.

7 References

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