RE: SOURCE

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NÄSTA GENERATIONS KOLDIOXIDNEUTRALA AVLOPPSRENINGSVERK-
CarbonNextGen

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RE:SOURCE

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CARBON NEUTRAL NEXT GENERATION WASTEWATER TREATMENT PLANTS-CarbonNextGen

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Preface

In face of climate change and other environmental impacts caused by human activities, innovative circular approaches are required on our path towards a more sustainable society. The presented project is our contribution related to the most significant activity to reduce societies negative impact on the environment: municipal sewage treatment. The report aims to demonstrate a new approach for a carbon neutral and circular wastewater treatment. Municipal organic wastes are transformed into functional carbon sources and high-value products securing the optimal resource recovery of these organic wastes and thereby contributing to a circular economy. Basic and applied research performed by collaborating organisations presents an approach for a carbon neutral wastewater plants closing the loop.

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Sammanfattning

Det övergripande målet för detta projekt var att demonstrera en nollavfallsstrategi som förvandlar två viktiga kommunala organiska avfall till funktionella kolkällor (VFA - volatile fatty acids (flyktiga fettsyror)) för nya högvärdiga produkter för att säkerställa optimal resursåtervinning och avfallshantering.

Resultaten visar att VFA-produktion från avloppsslam och externt organiskt avfall är möjlig under alkaliska förhållanden med pH 10 och 9, samt under surt pH 5 och vid ingen pH-kontroll. VFA-produktionen under alkaliska förhållanden med pH 10 uppnådde en maximal VFA-koncentration på 30 300 mgCOD/L och det högsta VFA-utbytet (815 mgCOD/gVS) med ättiksyra som den dominerande VFA, men produktionen minskade vid långtidskörning på grund av hög alkalinitet. Reaktorer med surt pH och utan pH-kontroll uppnådde maximala VFA-halter på 17 200 respektive 25 500 mgCOD/L och de högsta VFA-utbytena på 520 respektive 681 mgCOD/gVS med kapronsyra som dominerande VFA. Kapronsyra är en mer värdefull VFA-typ som används i olika applikationer inklusive antimikrobiella fodertillsatser och jetbränsleprekursorer. Den bakteriella kartläggningen antyder att de mikrobiella grupperna var beroende av reaktorns pH. Uppskalning till större pilot på 2 m^3 visade lovande resultat även utan pH-kontroll med högsta VFA-produktion på 21 100 mgCOD/L, men prestandan påverkades av substratets variation.

VFA-rika utflöden från alla reaktorer konstaterades vara goda kandidater som extern kolkälla för denitrifikationsprocessen med högre hastighet (12,6 -13,9 NOx-N/(g VSS h)) än båda metanol (5 mg NOx-N/(g VSS h)) och ättiksyra (10 mg NOx-N/(g VSS h)) som vanligtvis används som en extern kolkälla. Sammansättningen av VFA i det VFA-rika utflödet var en viktig bidragande faktor för deras effektivitet som kolkälla. VFA som härrör från avfallsströmmar har visat sig vara lovande substrat för PHA och anaerob nedbrytning kan utformas enligt önskad typ av PHA. Den högsta PHA-koncentrationen (3,3 g/L) erhölls med VFA-utflöde rik på kapronsyra, medan den högsta PHA-halten (43,5% vikt/vikt) uppnåddes med VFA rik på ättiksyra. De blandade mikrobiella kulturerna anpassade sig till VFA-avloppskompositionen och de konsumerade fettsyrorna i sin helhet. PHA-produktionen som använde sig av en kolkälla från avfallsströmmar var jämförbar med produkter som rapporterats från blandade kulturer, vanligtvis med syntetiska kolkällor och processer med striktare kontroll av pH och temperatur.

Den miljömässiga hållbarheten vid produktion av VFA och användning i produkter som ersätter andra fossilbaserade alternativ visades med hjälp av LCA-undersökning. Genom att undersöka klimatpåverkan av VFA-produktion och användning som kolkälla vid avloppsvattenrening i två skalor med produktion och användning av konventionella kolkällor illustrerades fördelarna med den presenterade resursåtervinningsmetoden. Den globala uppvärmningspotentialen för produktion av VFA och dess användning som kolkälla visade sig vara betydligt lägre än respektive påverkan från produktion och användning av fossilbaserad metanol.

CarbonNextGen har utvecklat ett nytt tillvägagångssätt för kolneutrala reningsverk. Kärnteknologin är en förbättrad rötningsprocess som behandlar både avloppsvatten och slam och skapar värde genom att producera energi och kommersiellt värdefulla fettsyror och bioplaster. CarbonNextGen-teamet planerar att ansöka i den kommande utlysningen för att fortsätta att tillämpa det föreslagna systemet i verkligheten genom samarbete mellan avloppsreningsverk i Stockholmsregionen. Den föreslagna biobaserade tekniken kan tas ut på marknaden som en kombination av produkter och tjänster som kan integreras i befintliga avloppsreningsverk inom en snar framtid.

Summary

The overall aim of this project was to demonstrate a zero-waste strategy that turns two important municipal organic wastes into functional carbon sources (VFA - volatile fatty acids) for new high-value products to ensure optimal resource recovery and waste management.

The results show that the VFA production from sewage sludge and external organic waste is feasible under alkaline conditions of pH 10 and 9, as well as under acidic pH 5 and no pH control. The VFA production under alkaline conditions of pH 10 achieved a maximum VFA concentration of 30300 mgCOD/L and the highest VFA yield (815 mgCOD/gVS) with acetic acid as the dominant VFA, but there was a decline in the production in the long run due to high alkalinity. Reactors operated under acidic pH and with no pH control achieved maximum VFA of 17200 and 25500 mgCOD/L and the highest VFA yields of 520 and 681 mgCOD/gVS, respectively, with caproic acid as dominant VFA. Caproic acid is a more valuable VFA type which is used in diverse applications including antimicrobial animal feed additives and jet fuel precursors. The bacterial mapping suggest that the microbial community was dependent on the pH of the reactor. Scaling up to bigger pilot of 2 m^3 showed promising results even with no pH control with highest VFA production of 21100 mgCOD/L, however, the performance was influenced by variability of the substrate.

VFA-rich effluents from all of the reactors were observed to be good candidates as a carbon source for the denitrification process with higher rate (12.6 -13.9 NOx-N/(g VSS h)) than both methanol (5 mg NOx-N/(g VSS h)) and acetic acid (10 mg NOx-N/(g VSS h)) used as an external carbon source. The composition of VFA in the VFA-rich effluent was a major contributing factor for their efficiency as carbon source.

VFAs derived from waste streams have shown to be promising substrate for PHA and anaerobic digestion can be designed according to the desired type of PHA. The highest PHA concentration (3.3 g/L) was obtained with VFA effluent rich in caproic acid, while the highest PHA content (43.5 % w/w) was attained with VFA rich in acetic acid. The mixed microbial cultures adapted to the VFA effluent composition and they consumed the fatty acids in their totality. The PHA production using a carbon source from waste streams was comparable to contents reported from mixed cultures usually using synthetic carbon sources and processes with more strict control of pH and temperature.

The environmental sustainability of the production of VFA and use in products substituting other, fossil-fuel based alternatives was shown with help of LCA-study. By investigating the climate impact of the VFA production and use as carbon source in wastewater treatment at two scales with the production and use of conventional carbon sources, the advantages of the presented resource recovery approach are illustrated. The global warming potential of the production of VFA and its use as a carbon source were found to be considerably lower than the respective impact from production and use of fossil-based methanol.

CarbonNextGen has developed a new approach in carbon neutral wastewater treatment plants. The core technology is an improved fermentation process that treats both the wastewater and sludge and creates value by producing energy, and commercially valuable fatty acids and bioplastics. *CarbonNextGen* team is planning to apply for the upcoming call to continue to apply the proposed system in the field with the collaboration of wastewater treatment plants of Stockholm Region. This proposed bio-based technology could be taken to the market as a combination of products and services that can be integrated into existing wastewater treatment plants in near future.

1. Introduction and Background

Environmental sustainability is a goal for the 21st century and to accomplish it, transition to a circular economy, which includes resource recovery, reuse and recycling, is required. Resource recovery contributes to environmental sustainability by reducing the consumption of raw materials and decreasing waste. Resource recovery from waste streams, which is dealing to United Nations Sustainable Development Goal 6 and 11(UN, 2015), is a major research interest, further boosted by the alarming increase in worldwide pollution levels and climate change primarily caused by overuse of fossil fuels. Therefore, energy and feedstock demand should be accommodated by renewable resources. Most EU countries including Sweden are trying to increase the use of renewable feedstocks through a transition into bio-based products, e.g. through the CO_2 emission trading system induced by the Kyoto protocol, policy objectives concerning energy security, stimulation of renewable transportation fuels at the EU level, and the CO_2 tax on fossil fuels in Sweden (Gavrilescu and Chisti, 2005). Sweden's new climate policy framework aims to fulfil the Paris Agreement by an ambitious strategy. One of Swedish long-term climate goals is to achieve no net emissions of Greenhouse Gases (GHGs) by 2045.

Methane and CO_2, which are very well known GHGs, are end products of wastewater treatment processes. In Sweden, methane which is produced in Municipal Wastewater Treatment Plants (WWTPs) is commonly used in public transport as fuel. However, the last decision of Stockholm Municipality is that electric buses will be started to use in public transport instead of biogas buses by 2021. This puts high demands on the development of next generation wastewater treatment (NGWT) technologies to turn these carbon-rich waste streams into new bio-based chemicals and materials instead of biogas production. Thus, new approaches are required to achieve the paradigm shift to a bio-based, circular economy.

Short chain fatty acids (SCFAs), also known as volatile fatty acids (VFAs), are valuable bioproducts which can be produced from these carbon rich waste streams. VFAs are important building block chemicals with a greatly increasing market demand (Hatti-Kaul et al., 2007; Yang, 2007; Zacharof and Lovitt, 2013). VFA production is traditionally based on non-renewable petrochemical sources, causing serious negative health and environmental effects. Reported greenhouse gas (GHG) emissions from acetic acid production in the petrochemical industry are 3.3 t $CO_{2eq.}$/t for cradle-to-grave (by incineration without energy recovery). Given that in 2009, the EU committed to reducing its GHG emissions by between 80% and 95% by 2050 (Wesselink et al., 2010), this process needs to be replaced with one utilising renewable sources and bio-based production methods.

The global municipal sewage sludge production per day is estimated at 100 Mt (Milieu Ltd. et al., 2010). From municipal waste sludge, an estimated 2 Mt of acetate, 1.28 Mt of butyrate, 1.54 Mt of propionate and 0.96 Mt caproic acid could be recovered annually throughout the world (Garlapati et al., 2016; Salamah and Randall, 2020). The total potential national return from a sewage waste process is estimated at € 12.3 M/year (Allied Market Research, 2020; Market Research Future, 2020; Marketsandmarkets, 2018). On the other hand, municipal wastewater treatment plants

(WWTP) have requirements for nutrient removal and therefore implement advanced biological treatment. Both biological nitrogen and phosphorus removal required easily biodegradable organic carbon source which is crucial to process efficiency and performance. Methanol is most common external carbon source used for denitrification process in conversion of nitrate to nitrogen gas at WWTPs in Sweden and other countries. Many studies in lab- and pilot scale have been done searching alternative carbon sources and VFA could be interesting for application if we could recover it at WWTP. The COD demand for removal of nitrogen is around 4,5 kg COD/kg NO_3-N and the demand for biological phosphorus removal is 10 kg COD/kg P (Henze et al., 1997). A big part of nitrogen is removed using the internal carbon in wastewater and removal of extra 2-5 mg/l of nitrate needs to be covered by external carbon addition in most WWTPs. Moreover, most of the phosphorus in Swedish WWTPs is removed by chemical precipitation. If a projected carbon source need for Himmerfjärdsverket WWTP is scaled up based on the number of people which are connected to the plant, the world need for carbon source is estimated at 30 Mt COD/y. If all the phosphorus was removed by biological phosphorus removal to a level of 0,3 mg/l (which is almost impossible to reach by application of only biological phosphorus removal), the estimated additional 40 Mt COD/y would have been needed. This corresponds to roughly 75 Mt acetic acid per year for both nitrogen and phosphorus removal.

Moreover, there has been the need to pursuit of novel and sustainable materials to replace conventional petroleum-derived plastics in order to ensure environmental sustainability. Polyhydroxyalkanoates (PHAs) can be synthesized by microorganisms using carbon sources and are building blocks of bioplastic. Substrate properties directly affect PHA structure and composition (Lee et al., 1995). A more sustainable carbon source such as waste streams and/or waste derived substrates needs to be sought to replace the conventional petrochemical based plastic in the medium and long run. VFAs are viable candidates as low cost, waste derived carbon source for PHA generation, and part of the excess capacity of 2,600,000 tons of VFA can be used to meet the current global bioplastic demand of 1,850,000 tons by year (Lee et al., 2014; TimeofChange, 2009). However, the main two limitations in PHA production from waste derived sources are low PHA production efficiency and PHA extraction from biomass. In the literature there are several studies showed that feast/famine regimes and nutrient limitations increase PHA production in the biomass (Dias et al., 2006; Reis et al., 2003).

The project has focused on (1) Optimization of volatile fatty acids (VFAs) production through co-fermentation of sewage sludge and external municipal bio-waste while assessing the resilience of such a system in a long-term operation. (2) Assessment of usage of volatile fatty acids (VFAs) as carbon source in the denitrification process. (3) Production of polyhydroxyalkanoates (PHAs), which is a main component of bioplastics, from VFA-rich effluent streams by using a semi-synthetic microbial community. (4) Evaluation of the environmental sustainability of the entire process and comparison to methane production.

2. Methods

2.1 Work Package 2: Volatile fatty acid (VFA) production through co-digestion

2.1.1 Substrate and seed sludge for VFA production

Primary sewage sludge (PS) from the Hammarby Sjöstadsverk research facility (Stockholm, Sweden) was used. For the batch (0.5 L) and semi-continuous (15 L) experiments, only homogenized (at 71°C for 61 min) external organic wastes (OW), collected from Himmerfjärden wastewater treatment plant (WWTP) (SYVAB, Sweden), were used as co-digestion. However, in the big pilot reactor of 2 m^3, three different organic waste streams including homogenized OW and non-homogenized OW from the Himmerfjärden WWTP and homogenized OW from Scandinavian Biogas (Södertörn, Sweden) were used in different operational period.

The external OW from the Himmerfjärden WWTP consisted of alcohol and soda beverage, food, dairy, fruit, fat and oil wastes. The OW from the Scandinavian Biogas is made up of food waste from households, school kitchens, restaurants, the grocery trade and the food industry and other organic waste from commerce and industries. Digested sludge taken from a full-scale anaerobic digestor (Henriksdal WWTP, Stockholm, Sweden) was used as seed sludge.

2.1.2 Batch experiments

Batch experiments were carried out using an automatic methane potential test system (AMPTS II, Bioprocess Control, Sweden) with biogas measurement unit and 500 mL glass reactors. The amount substrate was maintained at initial total chemical oxygen demand (COD) concentration of 15 g/L, whereas the initial amount of seed sludge set at VS of 10 g/L. Co-digestion was carried out with OW proportion of 0%, 25%, 50%, 75%, 100% as total COD. Thus, 0% OW and 100% OW represent mono-digestion of PS and OW respectively. All experiments were carried out in triplicates and at initial pH of 5 and 10. The pH was not controlled throughout the experiment. Temperature was controlled at 35°C.

2.1.2 Semi-continuous operation and microbial community analysis

2.1.2.1 Semi-continuous operation

Based on the results of the batch study, there were further experiments in semi-continuous mode in long term to determine the resiliency of the VFA production system. VFA productions were done in three parallel reactors operated with different pH control strategies, in a semi-continuous feed mode at 35°C. The feed consisted of 70% and 30% by volume of PS and OW, respectively. The study was carried out with three identical reactors (see Figure 1) set at acidic (pH 5), alkaline condition (pH 10 and 9), and no pH control with total volume of 15 L and working volume of 10 L. The acidic (pH 5) reactor was operated for 184 days and the other two reactors were operated for 315 days. The hydraulic retention time (HRT) was set at 7 days throughout the experiment with the exception of day 123-154 for no pH control reactor where the HRT was set at 10 days.
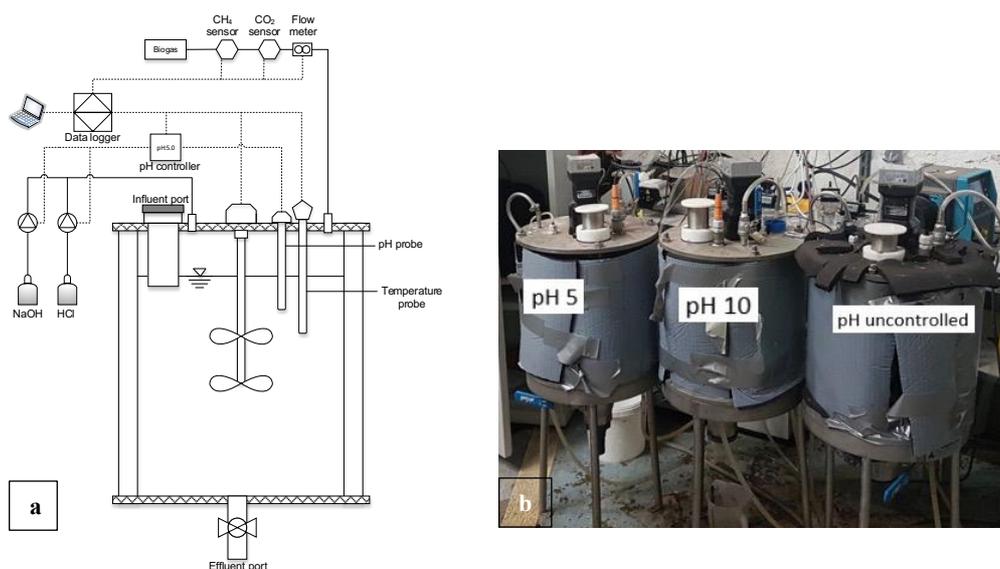
Figure 1. a) Schematic of the small pilot scale reactors used for the semi continuous experiments. b) Photos of the parallel fermenters. (Photo: Binyam Bedaso)

2.1.2.2 Microbial community analysis

Sludge samples from the semi-continuous reactors were taken on selected for microbial analysis. Since the pH of no control reactor was almost the same as pH 5, sludge samples were taken for pH 10 reactor and no pH control reactor. Total DNA of the sludge samples were extracted using NuceleoSpin Soil DNA kit (Macherey-Nagel, Germany) in accordance with manufacturer's protocol. PCR amplifications of the bacterial and archaeal 16S rRNA genes were carried out using primers 515F (GTGYCAGCMGCCGCGGTAA)-806R (GGACTACNVGGGTWTCTAAT) sequenced on Illumina MiSeq (300 bp paired-end, Illumina, Inc, San Diego, CA, USA) at the National Genomics Infrastructure (NGI) Sweden in SciLifeLab (KTH-Royal Institute of Technology, Sweden).

Bioinformatic analysis of the raw sequencing data was carried out using QIIME2 (Bolyen et al., 2019). SILVA database was used for the taxonomy assignments for bacteria and archaea (Bokulich et al., 2020).

2.1.3 Big pilot-scale reactor operation

From the results of the small pilot-scale reactors, the no-pH control reactor operation has proven to be optimal in the long term and therefore it was scaled up in a 2 m3 reactor as seen in Figure 2. The working volume of the reactor was 1.5 m3 and the reactor was operated at HRT of 7 days. All operation conditions are similar to the small pilot plant. The effect of substrate variability on the VFA production system was assessed by using different organic waste in the feed. Different kinds of organic waste as described in Section 2.1.1 were used. They included homogenised organic waste and non- homogenised from Himmerfjärden WWTP (SYVAB, Sweden) and homogenised organic waste from Scandinavian biogas. The four periods were studied based on the OW kind in the substrate. They were periods; I- PS and homogenized

OW from Himmerfjärden WWTP; II- PS and non-homogenized OW from Himmerfjärden WWTP; III-only PS; and IV- PS and homogenized OW from Scandinanvian Biogas.

Figure 2: Big Pilot Reactor for VFA Production (Photo: Isaac Owusu-Agyeman)

2.2 Work Package 3: The use of VFA-rich liquids as external carbon source for denitrification

2.2.1 Batch denitrification tests

To study the application of VFAs as an external carbon source in denitrification process, the manometric tracking method was used in lab-scale. VFA-rich effluent from the 10 L semi-continuous reactors were used along with traditional carbon sources including methanol, acetate as sole external carbon source with various C/N ratio in denitrification batch test. Furthermore, denitrification batch test using chemical tracking method (known as nitrate uptake rate-NUR test) was conducted to compare with manometric tracking method.

An airtight glass bottle (batch reactor) with a working volume of around 320 mL, and GMH 5150 manometric measuring device from Greisinger electronic GmbH (Regenstauf, Germany), featured with a data logger were used for the manometric tracking method. Program GSOFT 3050 was used to which GMH 5150 can log and export data.

The experiments were conducted with purpose to compare VFAs with other carbon sources and find optimum C/N ratio, generating the highest potential denitrifying activity (tests A-F). Since it has been proven that the feasibility and high-performance of VFAs as external carbon source, the tests G-I were conducted in order to further investigate denitrification capacity of VFAs produced from three different pH-controlled digestion reactors at Hammarby Sjöstadsverk (at pH of 10, pH of 5, and with no-pH control9. The experimental plan is presented in Table 1.

Table 1. Overview of experiment plan for batch denitrification tests

Aim	Test Group	C/N	External carbon source	Method	Number of Replicates
Denitrification tests with different carbon source and initial C/N ratio	A	7.5	Methanol, VFAs effluent produced from none pH-controlled digestion reactor (R3), Acetate sodium	Manometric tracking method	Two replicates
	B	6			
	C	4.5			
	D	3			
	E	1.5			
	F (control)	0	None		
Denitrification tests with VFAs produced from different pH-control digester reactors	G	4.5	VFAs effluent acquired from digestion reactor operated at pH of 10, pH of 5, and another without pH control respectively (R1,R2,R3)		
	H				
	I			Chemical tracking method	
Comparison of manometric- with chemical tracking method	J		VFAs effluent is acquired from digestion reactor operated at pH of 10 (R2)	Manometric tracking method & Chemical tracking method	Three replicates

The VFA-rich liquids were taken on day 100 of the semi-continuous pilot plant operation and tested for its denitrification potential in comparison with other conventional carbon sources for denitrification, specifically acetate and methanol, using the manometric tracking method. Denitrification tests with VFA-rich effluent from no pH control reactor were done at different carbon/nitrogen (C/N) ratios (from 0 to 7.5). Specific denitrification rate (mg NOx-N / g VSS·h) was calculated from equation 1:

$$K_N = \left(a \cdot \frac{M_{N_2} \cdot V_{HS}}{RT}\right) \cdot \left(\frac{1}{60 \cdot X_{VSS}}\right) \qquad \text{Eq.(1)}$$

Where:

K_N - Specific dentification rate
a - Slope value of the curve calculated with linear regression method describing changes of pressure in the batch reactor as the function of time
M_{N_2} - Molecular weight of nitrogen gas
V_{HS} - Head space volume in the batch reactor
R - Universal gas constant
T - Temperature
X_{VSS} - Amount of biomass in the batch reactor

2.2.2   *Continuous denitrification experiments:*

Denitrification using VFA-rich effluents from reactors operated under alkaline condition (pH 10) and no pH control as external carbon source were carried two identical glass reactors with a total capacity of 15 litres were used. The VFAs were taken from the reactors have a fill volume of ca. 10 litres, thereof 2 litres carriers of the type *AnoxKaldnes K5*, resulting in a filling fraction of 20 %. The influent wastewater was nitrified outflow of an upflow anaerobic sludge blanket (UASB) reactor treating municipal wastewater. The nitrate, nitrite, ammonium nitrogen and soluble COD concentration in the influent wastewater was 31 ± 4 mg NO_3-N/L, 2.1 ± 1.3 mg NO_2-N/L, 7.9 ± 2.8 mg NH_4-N/L and 50 ± 14 mg/L, respectively. To assess the performance of the denitrification MBBRs, the concentration of NO_2-N and NO_3-N was evaluated as NO_3-N equivalents (NO_3-N_{eq}). Therefore, the required oxygen equivalent to reduce 1 gram of NO_2-N divided by the required oxygen equivalent to reduce 1 gram of NO_3-N was used as conversion factor. With the oxygen equivalent of 1.71 g O_2/g NO_2-N and 2.86 g O_2/g NO_3-N, the measured concentration of NO_2-N could be converted into NO_3-N_{eq} by multiplying with a conversion factor of 1.71/2.86 (Tchobanoglous et al., 2014).

2.3 <u>Work Package 4: Polyhydroxyalkanoate production using effluents rich in volatile fatty acid as substrate</u>

Volatile fatty acids (VFA) produced by anaerobic digestion have been used as the carbon source to produce polyhydroxyalkanoates (PHAs). The produced VFAs described in section 2.2 were used for PHA production. The VFA effluent (VFA_2) obtained from the fermentation at pH 10 was rich in acetic acid (76.1% acetic acid), while at no pH control resulted in a VFA effluent (VFA_1) rich in caproic acid (43.5% caproic acid) (Table 2). The final VFA concentrations obtained from the anaerobic digestion every week were not always constant and for this reason, the VFA content was adjusted to a final concentration of 29.5 g/L, maintaining the average composition of the different short fatty acids. The content of ammonium in the VFA effluents was 1143 ± 151 mg/L NH_4^+ and 520 ± 113 mg/L NH_4^+ for the VFA rich in acetic acid and rich in caproic acid respectively. The effluents were filtered (200 μm) and kept at 4°C until further use.

Table 2. Composition of volatile fatty acids produced by anaerobic digestion at pH no control (VFA_1) and pH 10 (VFA_2).

Volatile fatty acid	Bioreactor pH no control VFA_1 (% w/w)	Bioreactor pH 10 VFA_2 (% w/w)
acetic acid	31,0	76,1
propionic acid	0,0	15,7
isobutyric acid	0,0	0,0
butyric acid	19,0	3,3
isovaleric acid	6,5	3,8
valeric acid	0,0	0,2
caproic acid	43,5	0,2
n-heptanoic acid	0,0	0,6

2.3.1 Biomass enrichment in Sequential Batch Reactors

Cultivations were performed in triplicate for each VFA effluent and in sequential batch reactors (SBR) of 5 L with a working volume of 2 L. The bioreactors were inoculated with aerobic sludge from an activated sludge process at Hammarby Sjöstadsverk, Stockholm, Sweden. The enrichment of the cells was designed with three phases: (1) a feast phase with all the nutrients in excess to allow cell growth, (2) a nitrogen-famine phase to allow PHA accumulation and (3) a nitrogen-carbon-famine phase where the cells that accumulated PHA can use it as an energy reservoir to survive the famine phase and be predominant in the next cycle. The SBRs were operated in cycles of 12 h with a hydraulic retention time (HRT) and solids retention time (SRT) of 24 h. At the end of each cycle, half of the volume of the reactor (1 L) was replaced with 865 mL of minimal medium and 135 mL of VFA effluent in order to have an initial VFA concentration of 2 g/L. The minimal medium contained: 5.4 g/L K_2HPO_4, 2.4 g/L KH_2PO_4, 10 mg/L thiourea to inhibit nitrification, 2 mL/L trace elements (1000x), 2 mL/L $MgSO_4$ (1M) and a variable amount of NH_4Cl depending on the ammonium content of the VFA effluent in order to have an initial NH_4Cl concentration of 5 mM. The trace elements solution consisted in: 0.8 g/L $CaCl_2 \cdot 2H_2O$, 9.7 g/L $FeCl_3 \cdot 6H_2O$, 0.4 g/L $ZnSO_4 \cdot 7H_2O$, 0.4 g/L $MnSO_4 \cdot H2O$, and 10 g/L Na_2-EDTA. Agitation and airflow were supplied. Temperature and pH were not adjusted during the cultivations. The average temperature in the bioreactors were around 21°C and the average pH was approximately 8. The reactors were cleaned every 14 days to avoid excessive biofilm formation. The enrichment phase was performed for 50 days and samples were taken three times per week to measure TSS, VSS, ammonium content, VFA concentration and PHA and also to sequence of DNA samples for bacterial characterization. Bacterial community analysis was done as described in Section 2.1.2.2.

2.3.2 PHA accumulation (nitrogen-limitation fed-batch)

After 50 days of enrichment, the cells were used in aerobic fed-batch cultivations to allow a higher PHA production. The fed-batch was performed in the same bioreactors and in triplicate as in the enrichment phase. The pH and temperature were not controlled; however, the average values were similar to the enrichment phase (around 21 °C and pH 8). The dissolved oxygen was kept above 1.8 mg/L by increasing the airflow or stirring to avoid oxygen limitation. At the beginning of the cultivation, 1 L of the enriched cells were mixed with 865 mL of fresh salt medium and 135 mL of VFA effluent and the initial concentrations were 2 g/L VFA and 5mM NH_4Cl. The experiments were performed with two phases: a batch phase (5 h) and a VFA feed phase (fed-batch), where nitrogen was the limited nutrient (48 h). The cultivations were performed with a constant feed and beside the VFA effluent, no other nutrient was added to the bioreactors. Samples to measure VFA concentration, PHA content, VSS, TSS and NH4+ were taken regularly during the whole experiment.

### 2.4 Work Package 5: Sustainability Analysis

A primary Life Cycle Assessments (LCA) was performed in order to get a first assessment of the environmental performance of the zero-waste strategy that turns sewage sludge and external organic waste into functional carbon sources that can be used for the production of high-value products. In order to compare the resource recovery and waste management as proposed in our project, the production of carbon sources used in the wastewater treatment was used as a case study. This partly because the addition of external carbon sources in water treatment is known to cause environmental impacts that are even increasing with continuously increasing requirements on nutrient removal from sewage. The example was further chosen because environmental data from conventional processes is available and data from our pilot-test was sufficient to model the environmental impacts. As conventional carbon sources both methanol and ethanol have been used as they are the most used carbon sources and a replacement of these by a VFA-based carbon source would imply the highest impact.

The following alternatives have been evaluated in the LCA:
1. VFA from primary sludge and external organic waste (OW) at acidic conditions: The case is based on the results of pilot reactor operation obtained in this project and described in chapter 3.1.4.
2. Fossil-based methanol: Methanol produced from steam reforming of natural gas, which is the traditional way for industrial methanol production today.
3. Renewable methanol: A new approach of producing methanol from CO_2 from flue gases and hydrogen from electrolysis.
4. Renewable ethanol: Ethanol produced from biomass in Brazil.
5. VFA from external organic waste (OW) in alkaline conditions: The case is based on the results of batch tests performed with the same substrate and methodology as described in chapter 3.1.1.

2.4.1 System boundaries

The study is a cradle-to-grave LCA, *i.e.* the climate impact from production of raw materials, transports to the production site, production of carbon sources, transport of the product to a WWTP and the use in a WWTP is included within the system boundaries. The results of this study are presented for one environmental impact category: global warming potential (GWP) with the unit kg CO_2-equivalents. Biogenic CO_2 is excluded. The functional unit was chosen as 1 kg of nitrogen removed in WWTPs. In **Figure 3** below, the system boundaries of the study are illustrated.
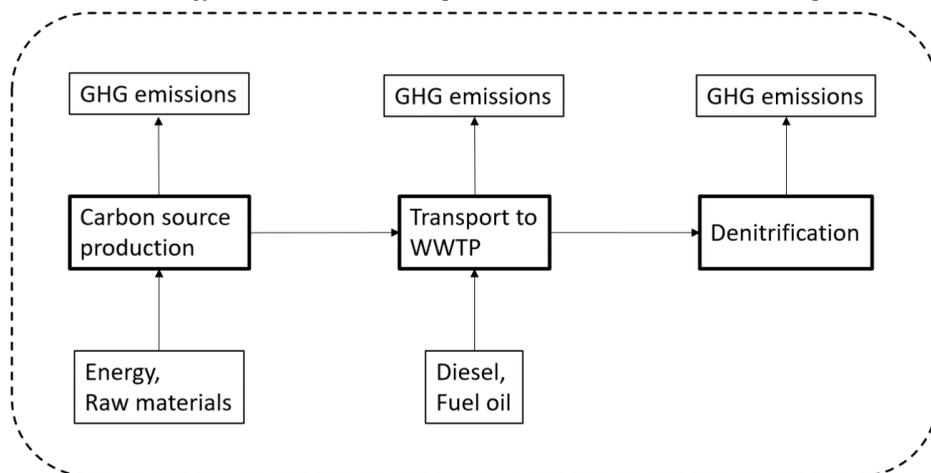
Figure 3. System boundaries of LCA.

The system boundaries were chosen deliberately to not include the alternative use of food waste in case it is not used for VFA-production. The food waste is usually anaerobically digested today. If part of the external organic waste is used for carbon source production, the biogas production may be altered which would affect negatively the net impact of the sludge fermentation technology. If system boundaries were extended in order to try to quantify this effect the same would need to be done even for other carbon sources, which would significantly increase complexity of the study and data on such a scenario was not available.

2.4.2 Assumptions

To facilitate the LCA calculations, some assumptions and simplifications had to be done. All COD contained in the carbon sources is assumed to be biologically available and is emitted as carbon dioxide in the denitrification stage at the wastewater treatment plant. In reality, some of the COD in the produced VFA-rich solution is not biologically available and cannot be used for denitrification. Moreover, part of the used COD in the denitrification process binds to biomass and is not emitted as CO_2 directly. Sludge production is different for different carbon sources and, therefore, there is a difference in how much COD is needed for denitrification of 1 kg of nitrogen when different carbon sources are used. These effects were however not taken into account in this LCA since it would significantly increase complexity of the evaluation and require more extensive studies on these aspects. It is further believed that these factors would not significantly effect the result of the comparison. The content

nitrogen in the produced VFA solutions was taken into account and the addition of COD was adjusted in order to get the net removal of 1 kg of nitrogen.

Because methanol and VFA can be produced locally, transports to wastewater treatment plants have been excluded. This is, however, not true for ethanol, which is assumed to be transported by ship from Brazil to Sweden.

Carbon dioxide used as a raw material for producing renewable methanol is considered burden-free, however emissions of CO_2 during the denitrification stage is assumed to be of fossil origin since the CO_2 used for production of methanol is captured from a cement plant. This is common practise in many LCA but in an alternative scenario emission of CO_2 could also be accounted for at the cement plant. Food waste used as a raw material for VFA production is also considered as burden-free.

It is assumed that electricity is used as the only energy source and covers the energy need for pumping, mixing and heating. In cases when an excess heat or district heating is available at site the environmental effect is lower due to lower specific greenhouse gas emission for these energy sources.

2.4.3 Life cycle inventory

The LCA modelling is done in the software GaBi, version 9.2.1 (Thinkstep, 2020a). LCA data are taken from databases Thinkstep (2020b) and Ecoinvent (Wernet et al., 2016).

Alternative 1. VFA produced from primary sludge and food waste in acidic conditions
Data of VFA production from primary sludge and food waste is based on the results of continuously operated pilot reactor run within the project. In the investigated scenario the system is scaled to a size sufficient for substitution of all of the external carbon source used at Himmerfjärdsverket WWTP. The calculations of energy requirement were done based on Elginoz et al. (2020) with some minor adaptions. The biggest difference between this case and case 4 is that the reactors are much bigger leading to a lower specific heat loss and that the energy recovery by heat exchangers is assumed leading to a lower heating requirement.

Alternative 2. Methanol production from steam reforming
LCA data of methanol production from steam reforming of natural gas is taken from the Ecoinvent database v. 3.6. The dataset has been adjusted to represent Swedish conditions, for example a Swedish electricity mix. Transport of methanol has been excluded since it is assumed that the methanol can be produced locally.

Alternative 3. Renewable methanol production
Data concerning methanol production from carbon dioxide and hydrogen is taken from Meunier et al. (2017). Data is based on process simulations in Aspen Plus software. The carbon dioxide is assumed to be free of burden from earlier life cycles but does however contribute to climate change due to it being sourced from cement production plants. The hydrogen is assumed to be produced from electrolysis and data is taken from Thinkstep database. Electricity is modelled as an average European electricity

mix. The steam used in the methanol manufacturing is assumed to be produced from natural gas. Transport of methanol from CO_2 and H_2 has been excluded since it is assumed that the methanol can be produced locally. Data on carbon dioxide emissions from respiration of methanol have been sourced from Tumlin et al. (2014). Since the carbon dioxide is assumed to be recovered from a cement production plant the carbon has a fossil origin and emissions of CO_2 during respiration does therefore contribute to climate change.

Alternative 4. Renewable ethanol production
LCA data of ethanol production from biomass is taken from the Ecoinvent database v. 3.6. The production is assumed to take place in Brazil and is thereafter shipped to Sweden to be used in WWTPs. Carbon dioxide emissions from respiration of ethanol are biogenic and does not influence the global warming potential.

Alternative 5. VFA produced from food waste in alkaline conditions
Data of VFA production from food waste is based on the lab scale tests performed with the same methodology and substrate as used in this project and discussed in chapter 3.1.1. A VFA-rich supernatant is produced which can be used as a carbon source in WWTPs. The VFA content in the supernatant is 1.7% and has approximately the same content of COD as methanol. The life cycle assessment of this case was done by Elginoz et al. (2020) and some data on energy use and CO_2 emissions was used for the LCA described in this report. The LCA is based on a scenario when a 1 m^3 fermentation reactor is operated.

Transport of VFA from food waste has been excluded since it is assumed that the VFA can be produced locally. Carbon dioxide emissions from respiration of VFA are biogenic and does not influence the global warming potential.

3. Results and Discussion

3.1 Volatile fatty acid production

3.1.1 Results Lab-scale experiments with different organic waste and primary sludge proportions

The VFA production increased with increase in the percentage of external organic waste (Figure 4). This is attributed to the increase in the biodegradable organics which were readily available for utilization by acid producing bacteria. It has been shown that co-digestion enhances the solubilization of hard-biodegradable organics which can improve VFA productivity.

The cumulative biogas volume was up to 175 NmL CH_4 at retention time of 7-day for the 0% OW experiment. Interestingly, the cumulative volume of biogas decreased with increase in the percentage of the organic waste. It can be inferred from the results that PS has higher tendency to form biogas even at acidic pH of 5 where methanogenic activities are minimal. This can be attributed to the composition of VFA formed by PS substrate. For experiments with initial pH of 10, the VFA concentration for the same trend as in pH 5 but the concentrations were higher (4000 to 8500 mg COD/L). Results are shown in the appendix (S1).
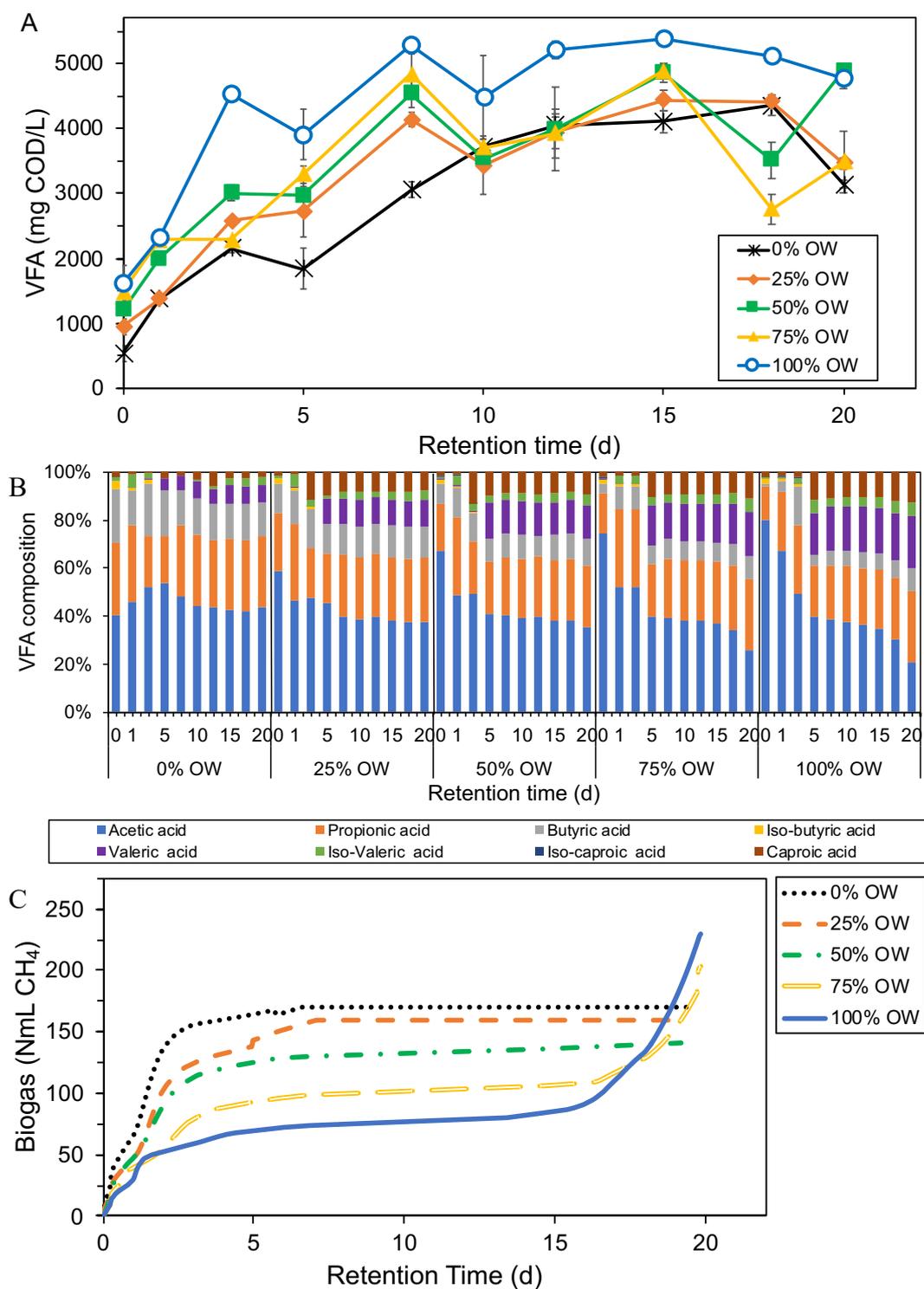
Figure 4. VFA concentrations (A), VFA compositions (B) and Biogas production (C) for lab-scale experiments conducted at initial pH of 5 with different proportions of external organic waste (OW). (Owusu-Agyeman et al., 2020)

3.1.2 Semi-continuous experiments

The results show that the VFA production for pH 5 and no pH control reactors initially increased until day 14 and began to decrease due to accumulation of undissociated acid (Figure 5 and Appendix S2). This effect was minimal in the alkaline reactor which operated at pH 10. However, the systems recovered due to acclimatization. The VFA productions for pH 5 and no pH control reactors were fairly stable after day 50 with a maximum VFA concentration of 17200 (Owusu-Agyeman et al., 2020) and 25500 mgCOD/L and highest VFA yields of 520 and 681 mgCOD/gVS, respectively. VFA production of pH 10-reactor kept increasing until day 121 with a maximum VFA concentration of 30300 mgCOD/L and the highest VFA yield (815 mgCOD/gVS). However, in the long run, unlike the pH 5 and no pH control reactors, the VFA production of the alkaline reactor reduced drastically to about 10000 mgCOD/L after day 121 due to high alkalinity (9900 mg $CaCO_3$). The results indicate how long-term operation study is important. Nonetheless, when the operation pH was reduced to 9 after the 267th day, it led to an increased in total VFA between 27000 and 32000 mg COD/L with an average of about 30500 mg COD/L. It is also important to mentioned that the concentration of propionic acid increased noticeably.

The effluents from the pH 5 and the no pH control reactors had similar VFA composition because the pH of the no pH control reactor was in the range of 5-5.7 at the stable conditions. VFA composition of the pH 10-reactor was about 75% acetic acid, while pH 5 and no pH control reactors produced mainly caproic acid (about 50%).
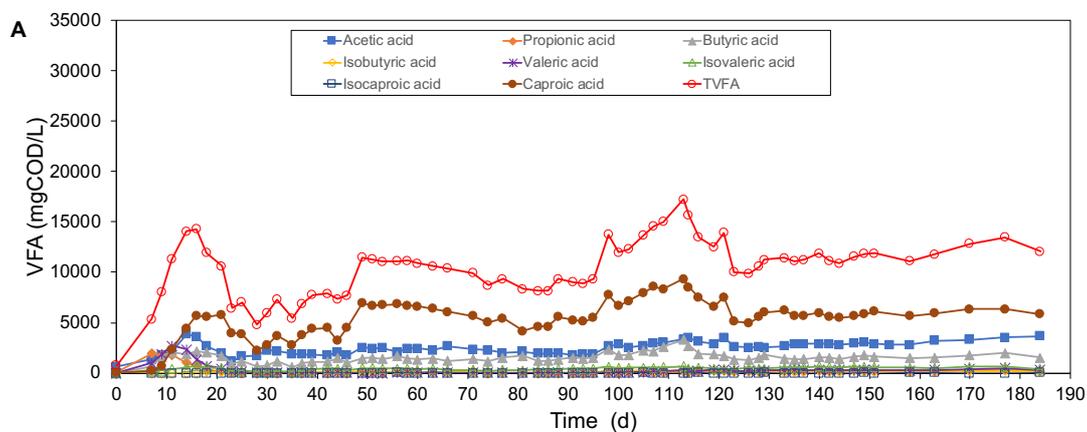
Figure 5. Total VFA production and individual VFAs from the reactors operated under acidic (pH 5) condition (Owusu-Agyeman et al., 2020). (Results for reactors operated under alkaline conditions and no pH control are shown in appendix S2)

3.1.3 Microbial population of the semi-continuous reactors

The bacterial population diversity showed that, at the phylum level, Firmicutes were the dominant bacteria when the reactor was operated at pH of 10 with relative abundance of 57 to 97% (Appendix S3). The second most dominant phylum was Proteobacteria with relative abundance of 1.1 to 14.5%, followed by Bacteroidota (0.7 to 10 %). The dominance of Firmicutes and Proteobacteria can be related to the fact both phyla are known to survive under high alkaline environment (Kalwasińska et al.,

2017). Firmicutes and Proteobacteria are known to be responsible for VFA production from mixed culture (Atasoy et al., 2019). In the family level, *Bacillaceae* (5-49%), *Proteinivoracales_uncultured* (8-39%), and *Carnobacteriaceae* (4-17%) were the most dominant bacterial populations between operation times 14 and 267 days when the reactor was operated under alkaline conditions. Lowering the pH to 9 resulted in drastic increase in the relative abundance of Bacteroidota to 55-60%. Members of Bacteroidota are known to be hydrolytic and acidogenic bacteria (Venkiteshwaran et al., 2016). Dysgonomonadaceae became the dominant family with relative abundance of 46-58% when the reactor was operated at pH 9. Principal component (PC) analysis of the bacterial diversity at phylum level showed that there was difference between pH 10 and pH 9.

Reactor operated with no pH control also had Firmicutes and Proteobacteria as the most bacterial phyla with relative abundance of 29-51% and 13-43%, respectively, during the operation periods between 14 and 315 days (see Appendix S4) In comparison with the reactor operated under alkaline conditions, the relative abundance of Firmicutes in the no-pH control reactor was lower, where as that of Proteobacteria was higher. For the same operation period, Bacteroidota had a relative abundance of 3-25%. Besides these three phyla, Actinobacteriota and Campilobacterota were also dominant in the reactor operated with no pH control with relative abundance of 0.7-16% and 1.4-11%. This shows that change in pH has influence on the bacterial diversity of VFA production system. PC analysis of the bacterial community in the phylum level showed that the start-up period 0-14 was grouped, whereas other periods were also grouped different. Day 46 was somehow different from the other period and seen as outliner.

3.1.4 Big Pilot scale plant

There was a scale up of the VFA production system to 2 m^3 and a working volume of 1.5 m^3 where the kind of external organic waste in the feed substrate was varied as explained in detail in Section 2.1.1. This helps to ascertain how the variability of the substrate which usually happen in a full-scale application can influence reactor performance. During the period I when, the reactor was fed with PS and homogenised OW from Himmerfjärden WWTP, there was an average total VFA of 15500 mgCOD/L with a maximum of 19000 mgCOD/L (Appendix S5). This was a little lower but comparable to what was obtained for the semi-continuous reactor where maximum of 25500 mgCOD/L was achieved, probably because the semi-continuous reactor was in smaller scale and the operational parameters were easier to control than in the bigger scale, where there were some operational problems with temperature control and pumping in the beginning. It needs to be mentioned that unlike the semi-continuous operation, there was no initial reduction in VFA production from acid accumulation effect, probably because the big pilot reactor was operated in the continuous mode therefore products are continuously removed from the reactor.

During period II, when the substrate was PS and non-homogenized OW from Himmerfjärden WWTP, there was a reduction in the VFA production with an average of 11900 mgCOD/L and a maximum of 17500 mgCOD/L. This reduction can be attributed to the homogenisation at 71ºC, which enhances VFA production. As to be

expected in period III when only PS was fed due to a shortage in OW supply, there was decrease in VFA production to an average of 10300 mgCOD/L. The results confirm our batch study which showed that the addition of OW significantly improve VFA yield from sewage sludge. Using non-homogenized OW from Scandinavian Biogas during period IV resulted in an increase in VFA production to an average of 17700 mgCOD/L and a maximum of 21100 mgCOD/L. The results show that OW from Scandinavian Biogas improves VFA production probably due to the high biodegradability of the external organic waste. Moreover, the SCOD of the period IV was significantly higher than other periods. There could be other organic products which amount have been formed.

3.2 <u>Testing VFA-rich Effluent as External Carbon Source for Denitrification</u>
3.2.1 Effect of carbon-nitrogen ratio on denitrification efficiency in short-term batch study

The VFA-rich liquid was taken on day 100 and tested for its denitrification potential in comparison with acetate and methanol. Denitrification was done in a batch test using the manometric tracking method with different carbon/nitrogen (C/N) ratios (from 0 to 7.5). Denitrification test results showed that the VFA-rich liquid achieved the highest specific denitrification among the three carbon sources (see Appendix S6). The results also showed that the COD/N ratio of 4.5 is sufficient for complete denitrification with a high denitrification rate. Thus, VFA production from waste has the potential to close the loop for carbon management of WWTPs.

3.2.2 Denitrification with different VFA-rich effluents in short-term batch study

To demonstrate that the VFA-rich effluents have potential to be used directly as external carbon sources in biological nutrient removal process, the VFA-rich liquids from the pilot scale reactor were tested for denitrification. The result shows that the VFA-rich liquids achieved the highest specific denitrification among the three carbon sources. The VFA-rich liquids from pH 10, pH 5 and no pH control reactors achieved a specific denitrification rate of 13.9, 12.6 and 12.6 mg NOx-N/(gVSS h), respectively, while the specific denitrification rate of acetate was 10 mg NOx-N/(g VSS h) (**Figure 6**). Methanol achieved specific denitrification rate of less than 5 mg NOx-N/(g VSS h), i.e. specific denitrification rate of the VFA-rich liquids were almost 3 times higher than that of methanol (Owusu-Agyeman et al., 2020). Methanol achieved the lowest denitrification rate because of the fact that methanol can be used directly by only methanol-assimilating bacteria while acetate and VFA-rich liquids are easily biodegradable (Liu et al., 2016). It would have been expected that acetate should have achieved higher rate than the VFA-rich broths since the VFA-rich liquids contained other organics which are harder to degrade than acetate. However, this was not the case and can be explained by the fact that a study has shown that VFA-rich liquids may contain cysteine which helps to reduce toxicity caused by reactive nitrogen generated by species of denitrifier during biological nutrient removal process (Su et al., 2016).
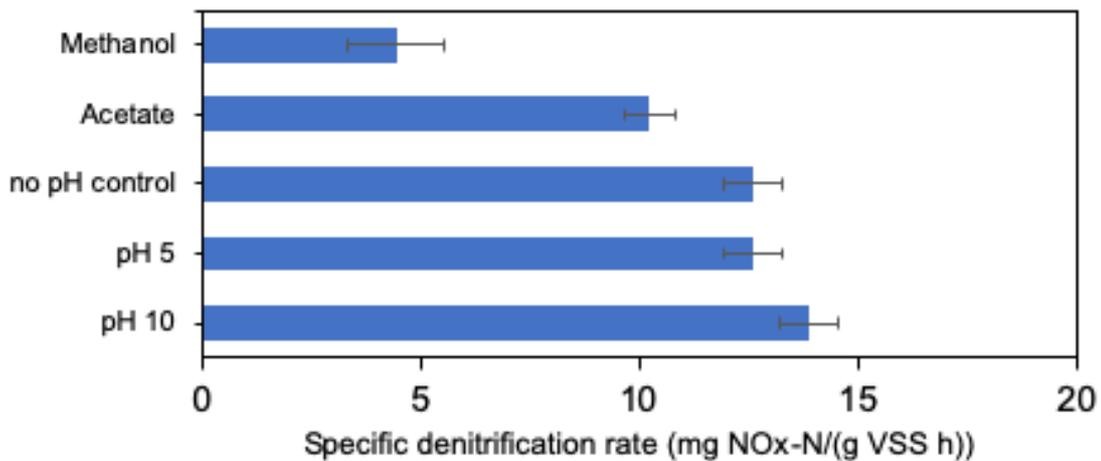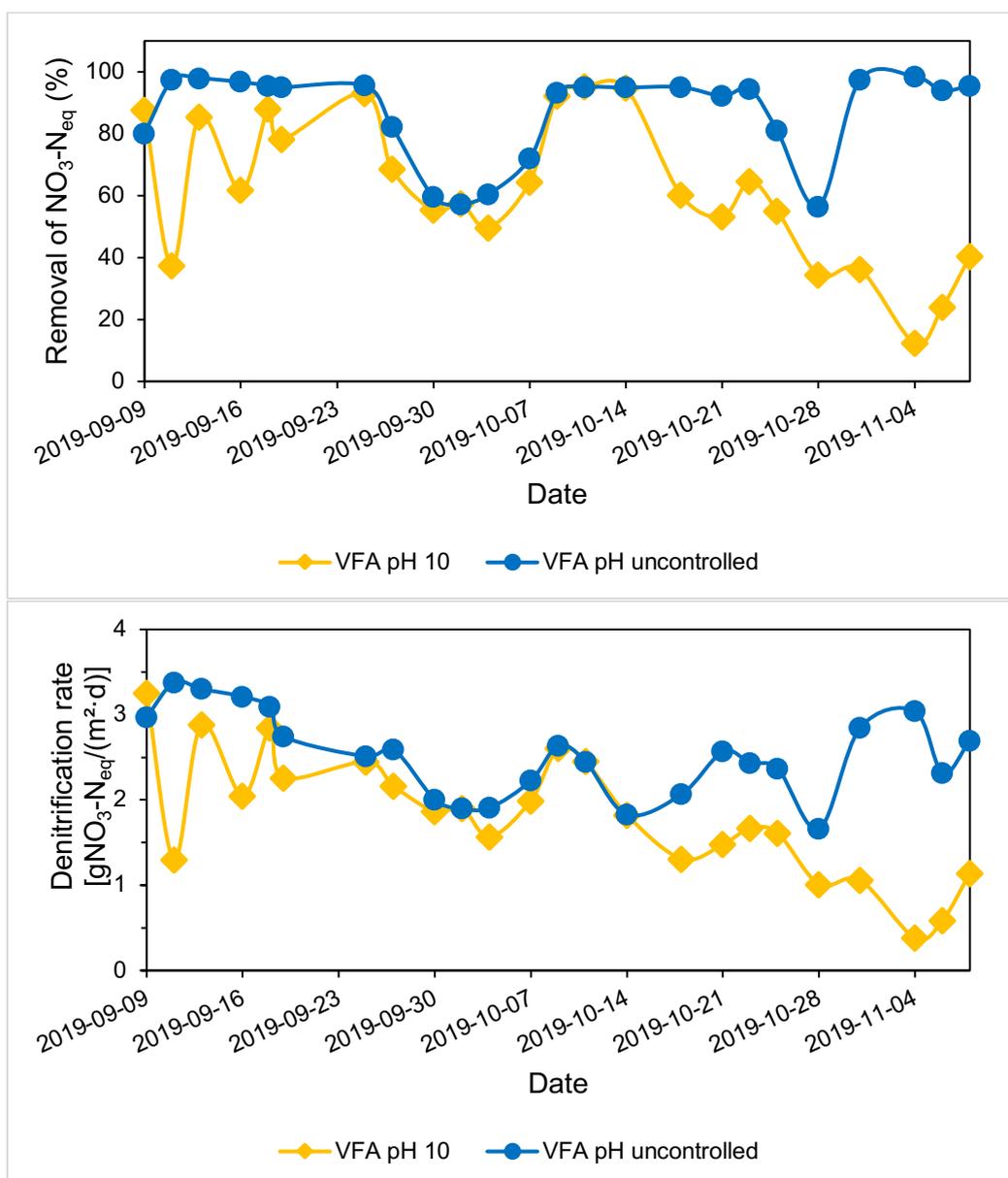
Figure 6. Denitrification rates with VFA-rich effluent as carbon source in comparison acetate and methanol. (adapted from Owusu-Agyeman et al., 2020)

3.2.3 Continuous denitrification with real nitrified wastewater

VFA-rich effluents from the 10 L semi-continuous reactors operated under alkaline conditions of pH 10 and with no pH control between operating days 210 and 267 and used as external carbon source for denitrification with real nitrified wastewater in moving bed reactor operated in a continuous mode.

Using VFA-rich effluents from the fermentation reactor operated under alkaline condition as carbon source for denitrification resulted in overall lower denitrification than from the reactor operated under no pH control (**Figure 7**). The average nitrite equivalent (NO_3-N_{eq}) removal efficiency for the reactors operated with VFA-rich effluent produced under alkaline pH (10) and no pH control were 62 ± 23 % and 87 ± 14 %, respectively. The difference can be attributed to the fact that the VFA proportion of the SCOD during the period when they were taken for denitrification experiments was lower for the effluent from the alkaline pH (10) reactor (0.34 ± 0.04 gVFA/gSCOD) than that from the reactor with no pH control (0.64 ± 0.04 gVFA/gSCOD).

In terms of denitrification rate, using VFA-rich effluent from the fermentation reactor operated with no pH control achieved denitrification rate of 2.5 g NO_3-N_{eq}/(m²·d), while the other VFA-rich effluent from the fermentation reactor operated at pH 10 reached a denitrification rate of 1.8 g NO_3-N_{eq}/(m²·d). The results show that both VFA-rich carbon sources are suitable for denitrification of municipal wastewater, however, the percentage of the VFA in the effluent is very important to achieve the needed results.

Figure 7. (A) Removal of nitrate equivalent and (B) denitrification rate during the continuous denitrification with real nitrified wastewater with the VFA-rich effluent as carbon sources

3.3 Polyhydroxyalkanoate production

3.3.1 Enrichment phase: The enrichment step is essential to obtain microbial mixed cultures with high PHA-storing capacity. In order to conduct a successful PHA enrichment process, feast and famine (nitrogen and carbon-nitrogen depletion) phases were performed under aerobic conditions during 50 days in cycles of 12 h. As mention before, two types of VFA effluents have been used and characterized (Table 2). Composition of VFA effluents varied depending on the pH used during the anaerobic digestion, where digestion at pH no control resulted in a VFA effluent rich in caproic

acid (VFA$_1$), while production at pH 10 resulted in a VFA effluent rich in acetic acid (VFA$_2$).

During the feast phase, ammonium consumption was quantified, where the yield of VSS per ammonium consumed ($Y_{VSS/N}$) for VFA$_1$ was only 7% higher than $Y_{VSS/N}$ of VFA$_2$ (Table 3). The growth rate was slightly higher when VFA$_2$ was used (0.09 1/h) compared with the microbial mixed culture enriched with VFA$_1$ (0.08 1/h) (Table 3). However, there was no significant difference between the growth rates and they were similar to the obtained in other researches using acetate or butyrate as carbon sources (Johnson et al., 2010; Marang et al., 2013).

Table 3. Consumption of ammonium and volatile fatty acids.

Bioreactor	VFA effluent	$Y_{VSS/N}$ (gVSS /g(NH$_4^+$))	r_{VFA} (gVFA/ L h)	q_{VFA} (gVFA/ gVSS h)	μ (1/h)	$Y_{VFA/VSS}$ (gVFA/ gVSS)
pH no control	VFA$_1$	3.16 ± 0.22	0.25 ± 0.02	0.26 ± 0.01	0.08 ± 0.01	3.59 ± 0.74
pH 10	VFA$_2$	2.94 ± 1.36	0.27 ± 0.05	0.21 ± 0.05	0.09 ± 0.02	2.59 ± 1.01

During the feast phase, the yield of VFA consumed per VSS ($Y_{VFA/VSS}$) obtained with VFA$_1$ was 28% higher than the $Y_{VFA/VSS}$ attained with VFA$_2$ (Table 3). At the end of each cycle, VFA samples were analyzed to confirm that no VFA was accumulated in the medium and eventually all acids were consumed. Comparing the VFA consumption rates with the obtained by Ahmadi et al., (2020), which used synthetic acetic acid as carbon source, it can be noticed that the yields are similar but the volumetric consumption rate of VFAs in this study were more than two-fold higher in either VFA$_1$ or VFA$_2$. Thus, the mixed cultures adapted to the VFA-substrates and the consumption in both cases was fast.

Microbial mixed culture using VFA$_2$ consumed mainly acetic acid with a specific consumption rate (q) 4 times higher compared to propionic acid and 6 times higher compared to the consumption of butyric acid (Table 4). Interestingly, microbial mixed culture enriched with VFA$_1$ showed a similar consumption of acetic acid and caproic acid and their consumption was simultaneously (Table 4). Both VFA effluents contained a small percentage of isovaleric acid but its consumption was very low, almost negligible. However, isovaleric acid was eventually consumed by the microorganisms since, at the end of the SBR cycle, there was no presence of this acid in the medium.

Comparing the acetic acid consumption of the enriched cultures, it can be noticed that the volumetric consumption rate obtained with VFA$_2$ was 30% higher than the obtained with VFA$_1$ (Table 4) This can be explained by the VFA composition (Table 2). As the concentration of acetic acid in VFA$_2$ was higher, the cells were exposed to a higher selective pressure of this acid, leading with the time to a better adaptation and consequently a higher volumetric consumption rate.

Table 4. Consumption rates and yield of each volatile fatty acid.

Bioreactor	VFA effluent	Volatile fatty acid	r (g/ L h)	q (g/ gVSS h)	$Y_{H/VSS}$ (g/ gVSS)
pH no control	VFA$_1$	acetic acid	0.11 ± 0.02	0.11 ± 0.01	1.52 ± 0.24
		butyric acid	0.06 ± 0.01	0.06 ± 0.00	0.88 ± 0.19
		caproic acid	0.10 ± 0.01	0.10 ± 0.00	1.43 ± 0.35
pH 10	VFA$_2$	acetic acid	0.16 ± 0.02	0.12 ± 0.02	1.53 ± 0.50
		propionic acid	0.05 ± 0.01	0.03 ± 0.01	0.44 ± 0.18
		butyric acid	0.03 ± 0.01	0.02 ± 0.00	0.22 ± 0.01

The composition of the carbon source affects directly the types of PHA obtained. During the enrichment phase, the microbial community enriched with VFA$_1$ produced only PHB, while cells enriched with VFA$_2$ produced PHBHV. Generally, fatty acids with even number of carbon atoms as acetate, butyrate or capronate, lead to the formation of (R)-3-hydroxybutyrate, while the acids with an odd number of carbons as propionate, valerate or isovalerate are considered precursors of (R)-3-hydroxyvalerate (Duque et al., 2014). 93.5% of the VFA$_1$ consisted of fatty acids with an even number of carbons and only 6.5% was isovaleric acid, which had a very low consumption rate during the SBR cultivations. In contrast, VFA$_2$ consisted of 79.6% fatty acids with even number of carbon atoms and 20.4% fatty acids with odd number of carbon atoms, mostly propionic acid (15.7%) (Table 2). The mixed culture enriched with VFA$_2$ accumulated PHBHV with an average composition of 94.2±1.9 %w/w (R)-3-hydroxybutyrate (3HB) and 5.8±1.9 %w/w (R)-3-hydroxyvalerate (3HV).

After 50 days of enrichment, the mixed culture produced 22.68 ± 7.77 (% PHA/VSS) when VFA$_1$ was used as substrate and 20.07±4.51 (% PHA/VSS) when VFA$_2$ was fed to the reactors. Moreover, the yield of PHA per grams of VFA consumed increased with the time and microbial culture fed with VFA$_2$ obtained a slightly higher yield than the obtained using VFA$_1$ (**Figure 8**).
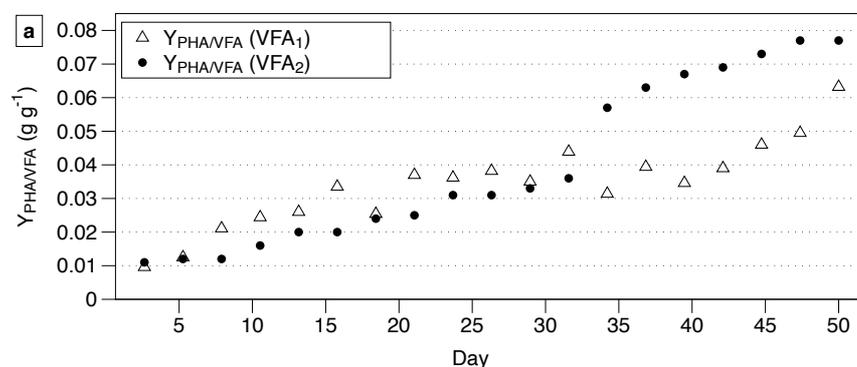
Figure 8 ▨ 3HB   :ion of PHA during the enrichment phase. (a) yield of PHA produced per VFA   umed.

▨ 3HV

3.3.2 PHA accumulation phase

To study the PHA accumulation capacity of the enriched microbial mixed cultures, fed-batch experiments were performed under nitrogen-limitation. The production of biomass was much higher for VFA_1 than VFA_2 and, at the end of the cultivation, the VSS concentration for VFA_1 was 3.5 times higher than the VSS for VFA_2. The percentage of PHA per active biomass evidently did not increase during the feeding phase, but as there are more cells, the concentration of PHA in the medium resulted in 3.3 g/L of PHA for VFA_1 and 1.3 g/L of PHA for VFA_2. When VFA_1 was fed to the reactors, the highest PHA content was 31.4 % (PHBHV/VSS) and, unlike the enrichment phase, both monomers (3HB and 3HV) were synthesized in the production phase (Figure 9a). The PHBHV obtained has a 3HB content of 97.1 ± 3.0 % (w/w), while 3HV solely stood for 2.9 ± 3.0 % (w/w) of the mass fraction of the PHA. In comparison, when VFA_2 was used as the substrate, the highest PHA content was 43.5 % (PHBHV/VSS) (**Figure 9**b). In this case, 3HB constituted of 94.8 ± 1.7 % (w/w), while 3HV accounted for 5.2 ± 1.7 % (w/w) of the mass fraction of the PHA. Generally, production of 3HV in the PHA polymer is desired since increases the flexibility, decreases stiffness and brittleness of the material and can expand the application area of the material. However, it can reduce crystallinity compared to PHB (Martínez-Sanz et al., 2014).

Even though higher PHA contents has been reported using microbial mixed culture, these investigations usually used synthetic carbon sources such as acetic acid under process with the strict control of pH and temperature (Serafim et al., 2004). Only few studies used VFAs from waste streams (Albuquerque et al., 2010). Taking into account only researches using waste stream to produce PHA by MMC, the PHA contents obtained in this study are comparable or higher than the reported before. For example, when heat pretreated waste sludge was used as a substrate, the obtained PHA content was 24.1% (w/w) (Liao et al., 2018), while when food waste was used to produce VFA rich effluents, the maximal PHA accumulation was 23.7% (w/w) (Amulya et al., 2015). Zhang et al., (2019) obtained similar PHA values (34.6 %w/w) when VFAs produced from primary sludge were used.

(a) VFA₁   **(b) VFA₂**
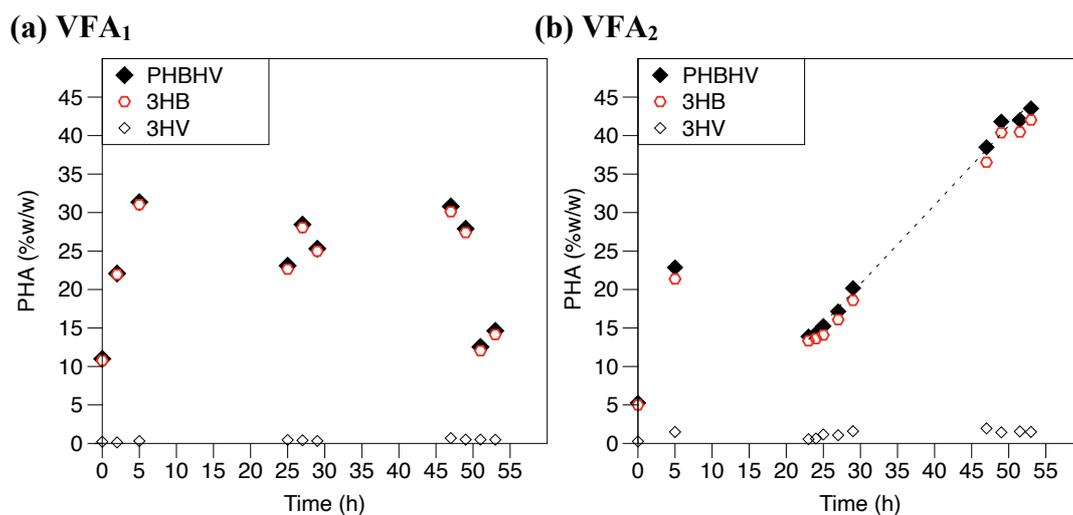
Figure 9. Accumulation of PHA during the production phase. The cultivations were performed in fed-bath mode, limiting the nitrogen substrate. (a) production of PHA

using VFA effluent rich in caproic acid (VFA$_1$) (b) production of PHA using VFA effluent rich in acetic acid (VFA$_2$).

3.4 Life Cycle Assessment (LCA)

The results of the carbon footprint are presented in **Figure 10** below. It is evident from the results that direct emissions of carbon dioxide from fossil carbon sources in the denitrification step at the WWTP is the largest contributor to the overall climate impact of the carbon sources. Because VFA and ethanol are produced from biomass, the carbon dioxide emissions does not contribute to the climate change. As described in the methods, carbon dioxide used for producing renewable methanol is considered burden-free but emissions of CO_2 from renewable methanol during the denitrification stage is accounted for since the CO_2 used for production of methanol is captured from a cement plant. One could argue that the use of captured CO_2 from a cement plant does not alter the total emissions of CO_2 as it would have been emitted at the cement plant otherwise. Presented results for renewable methanol in the figure below may thus also consider only production emissions, which would change the accounted impact for this alternative significantly.

Unlike the other carbon sources, ethanol needs to be shipped from Brazil to Sweden. However, this impact does not affect the carbon footprint to a large extent.
Steam reforming of natural gas to produce methanol has the largest carbon footprint out of the five studied alternatives, even if the denitrification step would be excluded.
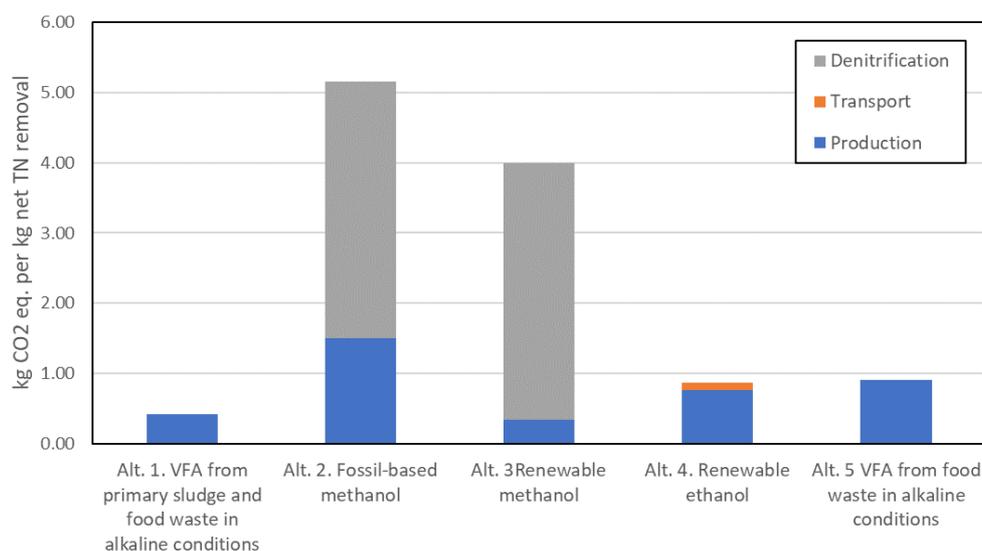
Figure 10. Results of the life cycle assessment of different carbon sources.

It is clear that denitrification using internally produced VFA at large scale (Alternative 1) gives the lowest greenhouse gas emissions. The reduced climate impact when going from smaller scale (Alternative 5) to larger scale (Alternative 1) also indicates a further potential to improve the VFA production from sludge and organic waste. However,

one might argue that use of bioethanol for denitrification increases consumption of other fossil fuels, e.g. gasoline since ethanol is used as gasoline substitute. In the same way use of COD in food waste and primary sludge for denitrification would probably decrease the biogas production. If biogas is applied as vehicle fuel (around 50% of all the biogas produced at Himmerfjärdsverket is upgraded and used as vehicle fuel) the decrease of biogas production could increase the gasoline consumption and, in that way, indirectly increase the greenhouse emissions. However, as today Himmerfjärdsverket is as many other WWTPs producing much more biogas than the market for vehicle fuel can use. The production of VFA from the same substrate may therefore not compete with the biogas production. Instead it may provide an addition reduction of environmental impacts as produced but not sold biogas would not have to be flared. The environmental sustainability of the production of VFA and use in products substituting other, fossil-fuel based alternatives appears obvious based on the initial LCA.

A more detailed LCA with access to data from long-term trials at a larger scale would be necessary to quantify all the various aspects presented including uncertainties. More data on the biogas production from the solid residue of the anaerobic fermentation would also be required. This is suggested to be investigated in future studies.

4. Conclusion, Benefits and Recommendations

Sewage sludge and external organic waste (including food waste) which are produced in each city in the world were used for recovery of VFAs as platform products for other bio-based applications. The compositions of the VFA produced from the waste streams were influenced by the operating pH and the mode of operation. Caproic acid, which is expected to grow at a compound annual growth rate (CAGR) of roughly 2.9% over the next four years, was the dominant VFA at acidic pH whereas acetic acid dominated when the reactor was operated under alkaline pH range. This was attributed to the fact the pH influenced the microbial community of the reactors. Application of the VFA-rich effluent as carbon sources for denitrification showed a promising result with the highest specific rates in comparison with conventional acetate and methanol. Moreover, the waste-derived VFAs were successfully used as substrates for PHA production. The highest PHA concentration (3.3 g/L) was obtained with caproic acid dominated VFA, while the highest PHA content (43.5 % w/w) was attained with acetic acid dominated VFA. The results also indicate that the VFAs derived from waste streams are a promising substrate for PHA and anaerobic digestion can be designed according to the desired type of PHA. Furthermore, composition of VFA not only affects the polymer storage but also the microbial community and substrate consumption. Hence, adaptive evolution of mixed cultures to the substrate is essential to achieve high consumption and production rates. In addition, the proposed fed-strategy can be used for a continuous PHA production process, from anaerobic fermentation to downstream processing, without the need for an accumulation stage. In **CarbonNextGen** proposal, project team describe the expected results as: *"Expected results include demonstration of a zero-waste strategy for municipal wastewater treatment plants and complementary high-value product alternatives to today's*

biofuel. The main outputs of the proposed project are: (1) verifying the optimised VFA production conditions for two selected municipal organic wastes, (2) enhancing VFA production from these two selected municipal organic wastes, (3) developing a methodology to apply VFA rich stream as external carbon source in denitrification process, (4) PHA production from VFA rich stream and (5) evaluation of the environmental sustainability of the two applications instead of or complementary to methane production. The project will close the loop in municipal wastewater treatment plants and deliver next generation wastewater treatment approach, which aims at carbon neutral system and circular economy."

As seen in the result section, all planned outcomes were achieved. By *CarbonNextGen*, circular city concept in terms of waste management was achieved in the lab-pilot scale. In the light of the findings of *CarbonNextGen*, boundary of carbon neutral next generation wastewater treatment plants as proof-of-concept is determined. The main aim of *CarbonNextGen* was to create value from waste by producing energy, VFA and bioplastic. VFA has a greatly increasing market demand as currently $400-800/t by petrochemical sources. This developed biobased technology could be taken to the market as a combination of products and services where we can optimize design and operational parameters of existing wastewater treatment plants as well as add system components and biological additives to increase production of VFA and PHAs. *CarbonNextGen* as next generation production technology is in up-scaling process in different scales and could be potentially transferred to full-scale application in 5 years. In *CarbonNextGen*, KTH, Syvab and IVL closely collaborate to develop this technology to reach market.

The next step is to upscale the concept and to apply this concept in the field (in real wastewater treatment plants). For this purpose, same project team is planning to apply to the next call of Re:source platform with representative of wastewater treatment plants in Stockholm. To achieve this consortium, after the submission of the final report, a last dissemination activity is planned to present the outputs of CarbonNextGen and the project proposal of *CarbonNextGen 2.0*.

5. Publication List

International Peer-Review Scientific Articles:
1) Mariel Perez-Zabaleta, Merve Atasoy, Kasra Khatami, Elsa Eriksson, and Zeynep Cetecioglu. Bio-based conversion of volatile fatty acids from waste streams to polyhydroxyalkanoates using mixed microbial cultures (2020) (in preparation): This study will summarize the capacity of mixed microbiome in wastewater treatment plants to turn VFA mixture to PHA and responsible species in the system.
2) Owusu-Agyeman, I., Balachandran, S., Plaza, E., & Cetecioglu, Z. (2020). Co-Fermentation of Municipal Waste Streams: Effect of Pretreatment Methods on Volatile Fatty Acids Production. Biomass and Bioenergy (Under review): This study summarize how pretreatment methods increase VFA prodution efficiency in fermentation of primary sludge and food waste.

3) Khatami, K., Perez Zabaleta, M., Owusu- Agyeman, I., & Cetecioglu, Z. (2020). Waste to bioplastics: how close are we to sustainable polyhydroxyalkanoates production? Waste Management. https://doi.org/10.1016/j.wasman.2020.10.008: It is a review paper to evaluate the current methods and market challenges fir bioplastic production from waste streams.

4) Owusu-Agyeman, I., Plaza, E., & Cetecioglu, Z. (2020) Production of volatile fatty acids through co-digestion of sewage sludge and external organic waste: effect of substrate proportions and long-term operation. Waste Management, 112, 30–39: This article presents the results from batch experiment and 10 L semi-continues reactor operation to produce VFA under pH 5 condition.

5) Atasoy, M., Owusu-Agyeman, I., Plaza, E., & Cetecioglu, Z. (2018). Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresource technology, 268, 773-786: This is a review article summarize current technologies and limitations on both production and recovery of VFAs from waste streams.

Thesis:

Owusu-Agyeman, I (2020). Recovery of organic carbon from municipal waste streams. TRITA-CBH-FOU-2020:23. (Licentiate thesis)

Elsa Eriksson (2020). Polyhydroxyalkanoate production from municipal waste streams. TRITA-CBH-GRU-2020:039. (Master thesis).

Doehler, C.M. (2020) "Volatile fatty acid production and application as external carbon source for denitrification. TRITA-ABE-MBT:2034 (Master thesis).

Chengyang, P. (2019). Application of volatile fatty acids from waste as an external carbon source for the denitrification process. TRITA-ABE-MBT; 19682. (Master thesis).

Bedaso, B. (2019) Volatile fatty acid production from co-fermentation of primary sludge and food waste without pH control. TRITA-ABE-MBT; 19691. (Master thesis).

Conference Papers:

Owusu-Agyeman, I., Plaza, E., & Cetecioglu, Z. (2019). Anaerobic co-digestion of sewage sludge and external organic waste: strategy to shift production from biogas to volatile fatty acids. 3rd IWA Resource Recovery Conference, September 8-12, 2019. Venice (Oral presentation)

Owusu-Agyeman I., Malovanyy A., Baresel C., Plaza E., Cetecioglu. Z. Production of volatile fatty acids from sewage sludge and food waste for denitrification: closing the loop for wastewater treatment plants. IWA Nutrient Removal and Recovery Conference (Virtual), Helsinki, Finland 1-3 September 2020 (Oral Presentation)

Owusu-Agyeman I., Malovanyy A., Baresel C., Plaza E., Cetecioglu. Z. Optimization of volatile fatty acids production from sewage sludge and food waste for up-scaling

purposes: closing the loop for treatment plants. 2020 Closed Cycles and Circular Society Symposium (Virtual). Waedenswil, Switzerland, 2-4 September 2020. (Poster)

Mariel Perez-Zabaleta, Kasra Khatami and Zeynep Cetecioglu. From waste to bioplastics: bio-based conversion of volatile fatty acids to polyhydroxyalkanoates. Accepted abstract for oral presentation in the Annual conference of microbiology society 2020, Edinburgh. Conference cancelled due COVID-19. DOI: 10.1099/acmi.ac2020.po0900

6. Project Communication

The project communication was achieved by different channels:
1) The project results were presented in different conferences and symposiums. Some of them were invited talks. Target groups were scientists and practitioners.
   - Cetecioglu, Z. Acidogenic bacteria as our co-workers for more sustainable production process. Open Conference of EuroMicropH. 12-14 February 2020, Lisbon, Portugal. Invited talk.
   - Cetecioglu, Z. Anaerobic Digestion and Beyond for the Circular Economy. VII BalticBiogasand Circular Economy Forum, September 22-23, 2020. Online. Invited talk.
   - Owusu-Agyeman, I., Plaza, E., & Cetecioglu, Z. (2019). Anaerobic co-digestion of sewage sludge and external organic waste: strategy to shift production from biogas to volatile fatty acids. 3rd IWA Resource Recovery Conference, September 8-12, 2019. Venice (Oral presentation)
   - Owusu-Agyeman I., Malovanyy A., Baresel C., Plaza E., Cetecioglu. Z. Production of volatile fatty acids from sewage sludge and food waste for denitrification: closing the loop for wastewater treatment plants. IWA Nutrient Removal and Recovery Conference (Virtual), Helsinki, Finland 1-3 September 2020 (Oral Presentation and Panel discussions)
   - Owusu-Agyeman I., Malovanyy A., Baresel C., Plaza E., Cetecioglu. Z. Optimization of volatile fatty acids production from sewage sludge and food waste for up-scaling purposes: closing the loop for treatment plants. 2020 Closed Cycles and Circular Society Symposium (Virtual). Waedenswil, Switzerland, 2-4 September 2020. (Poster)
   - Mariel Perez-Zabaleta, Kasra Khatami and Zeynep Cetecioglu. From waste to bioplastics: bio-based conversion of volatile fatty acids to polyhydroxyalkanoates. Accepted abstract for oral presentation in the Annual conference of microbiology society 2020, Edinburgh. Conference cancelled due COVID-19. DOI: 10.1099/acmi.ac2020.po0900
2) The project was presented nationally to Swedish stakeholders: VA-Mälardalen Cluster meetings/workshops, where representatives from Swedish wastewater treatment plants participated (21-22/11 2018, 27–28/8 2020).
3) The results were presented to the members in two COST action: CA17133 and CA18113 as MoU of Sweden. The target groups were scientist and practitioners.

4) The results were shared via Twitter and LinkedIn by hashtag *#CarbonNextGen*. The target groups were public, stakeholders and colleagues.

5) *CarbonNextGen* was nominated and then selected to **IVA's 100 Lista**. It was a big achievement for the project. The target groups are companies, industries and financiers.

6) After *CarbonNextGen*, a new sister project entitled Methodology for environmental sustainability assessment in the early design stage of a resource recovery system (*MENToR*) and supported by FORMAS has being coordinated by PI Zeynep Cetecioglu Gurol. In *MENToR*, the aim is to develop a new environmental assessment method for early design systems. *CarbonNextGen* provided most of the data to *MENToR* project and *MENToR* project provided many feedbacks in the early design steps of our proposed systems in terms of environmental burdens.

7) Based on *CarbonNextGen* outputs, a new H2020 application was done to the call FNR-12-2020 by the coordination of KTH. This new proposal, *NextGenVFA*, passed the first stage, and the second stage proposal was submitted in September 2020. *NextGenVFA* focuses on industrial waste streams in the scope circular cities and bioeconomy-

8) The project activities including the pilot equipment were presented in the guided tours at the R&D-facility Hammarby Sjöstadsverk with several hundred national and international visitor groups every year.

9) The project has been followed by both national and international researchers, Swedish PhD students (Water Research School), representatives from WWTPs

Planned Dissemination Conferences:

The project findings will be presented at a number of different national and international conferences, seminars and meetings. Some of these occasions such as the National Wastewater Conference have been postponed due to COVID-19 and venue date and place remain yet unclear. The same is true for other planned activities such as presentations of project results at the following conferences with accepted papers:

- Owusu-Agyeman I., Bedaso B., Pan C., Malovanyy A., Baresel C., Plaza E., Cetecioglu Z. (2020) Volatile fatty acids production and application in wastewater treatment plants: a pilot-scale study IWA World Water Congress & Exhibition. 18-23 October 2020 Copenhagen, Denmark (Postponed to 9 – 14 May 2021)

- Owusu-Agyeman I., Malovanyy A., Baresel C., Plaza E., Cetecioglu. Z. Volatile fatty acids production from sewage sludge and external organic waste for up-scaling purposes: pH effect and microbial dymamics. 5th IWA Specialized International Conference 'Ecotechnologies for Wastewater Treatment (ecoSTP) 2020' - June 22-26, 2020 - Milan, Italy (Postponed to June 21-25, 2021)

It is planned to present project results during 2021 at:

- Cluster Meetings with experts and WWTP-operators in the Mälardalregion.
- A dissemination seminar oriented towards representative from WWTPs' representatives as stakeholders.

- Summarizing paper with achievements of the *CarbonNextGen* project and presentation at NORDIWA Wastewater Conference in Gothenburg 28-30 September 2021.

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8. Attachments

Attachment 1: Administrativ Bilagor
Attachment 2: List of Publications, Submitted Manuscripts and Thesis
Attachment 3: Appendix which included non-published data