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Reconsidering hydrolysis kinetics for anaerobic digestion of waste activated sludge applying cascade reactors with ultra-short residence times

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ABSTRACT

Hydrolysis is considered to be the rate-limiting step in anaerobic digestion of waste activated sludge (WAS). In this study, an innovative 4 stages cascade anaerobic digestion system was researched to (1) comprehensively clarify whether cascading configuration enhances WAS hydrolysis, and to (2) better understand the governing hydrolysis kinetics in this system. The cascade system consisted of three 2.2 L ultra-short solids retention times (SRT) continuous stirred tank reactors (CSTRs) and one 15.4 L CSTR. The cascade system was compared with a reference conventional CSTR digester (22 L) in terms of process performance, hydrolytic enzyme activities and microbial community dynamics under mesophilic conditions (35 °C). The results showed that the cascade system achieved a high and stable total chemical oxygen demand (tCOD) reduction efficiency of 40–42%, even at 12 days total SRT that corresponded to only 1.2 days SRT each in the first three reactors of the cascade. The reference-CSTR converted only 31% tCOD into biogas and suffered process deterioration at the applied low SRTs. Calculated specific hydrolysis rates in the first reactors of the cascade system were significantly higher compared to the reference-CSTR, especially at the lowest applied SRTs. The activities of several hydrolytic enzymes produced in the different stages revealed that protease, cellulase, amino peptidases, and most of the tested glycosyl-hydrolases had significantly higher activities in the first three small digesters of the cascade system, compared to the reference-CSTR. This increase in hydrolytic enzyme production by far exceeded the increase in specific hydrolysis rate, indicating that hydrolysis was limited by solids-surface availability for enzymatic attack. Correspondingly, high relative abundances of hydrolytic-fermentative bacteria and hydrogenotrophic methanogens as well as the presence of syntrophic bacteria were found in the first three digesters of the cascade system. However, in the fourth reactor, acetoclastic methanogens dominated, similarly as in the reference-CSTR. Overall, the results concluded that using multiple CSTRs that are operated at low SRTs in a cascade mode of operation significantly improved the enzymatic hydrolysis rate and extend in anaerobic WAS digestion. Moreover, the governing hydrolysis kinetics in the cascading reactors were far more complex than the generally assumed simplified first-order kinetics.

Introduction

Waste activated sludge (WAS) is an inevitable by-product generated in biological wastewater treatment plants (WWTPs). Due to quantitative and qualitative extension of wastewater treatment, the annual WAS production has increased in the European Union during the last two decades, from 10 million tons in 2008 to 11.5 million tons in 2015, and is expected to approach 13 million tons by 2020 (Rorat et al., 2019).

Anaerobic digestion (AD) is a proven key technology for both stabilization of WAS and recovery of the biochemical energy stored in the sludge in the form of biogas. WAS usually contains complex particulate organics, such as proteins, polysaccharides, lignocellulosic matters, and fats (Gonzalez et al., 2018). Hydrolysis of WAS into soluble substrates is the first step in AD and is generally regarded as the rate-limiting step in this process (Appels et al., 2008). Therefore, conventional digesters using continuous stirred tank reactors (CSTRs) have to be operated

under prolonged sludge retention times (SRTs) exceeding 20 days for an acceptable WAS conversion.

To accelerate the conversion rate of WAS and decrease these long SRTs, process optimisation has been applied as well as the development of hydrolysis enhancement technologies, including thermal, chemical and enzymatic methods (Zhen et al., 2017). Enzymatic hydrolysis enhancement seemingly offers unique advantages compared to chemical or physical processes, as it neither causes generation of toxic substances, nor needs operations under extreme conditions, thus receiving an increased attention in the recent years (Gonzalez et al., 2018). Most of these studies focused on the direct addition of highly active hydrolytic enzymes into the digester (Yang et al. 2010) or on pre-fermentation by specific hydrolytic bacteria prior to AD (Agabo-Garcia et al., 2019). These proof-of-concept methods showed remarkable improvement in WAS hydrolysis and bio-degradation; however, full scale applications require a continuous purchase of enzymes and/or the need for preservation of specific biomass while working with poorly defined substrates.

Hydrolysis of organic matter during AD is performed by extracellular and/or membrane-bound hydrolytic enzymes (Kim et al., 2012). Enhancement of WAS hydrolysis also can be achieved by accelerating the reaction rates and/or increasing the activity of these hydrolytic enzymes instead of adding external hydrolytic enzymes or applying pre-fermentation. A commonly applied strategy is to perform WAS digestion under thermophilic (55 °C) conditions, which roughly results in a doubling of the enzymatic reaction rates compared to the commonly applied mesophilic (35 °C) conditions (Ge et al., 2011a). Nevertheless, decreased process performance was often observed under thermophilic conditions due to the accumulation of organic intermediates to a toxic level, or to a drop in pH (Kim et al., 2003), negatively impacting the actual enzymatic reaction rates. In addition, other constraints of thermophilic WAS digestion include higher energy requirement, poor effluent quality and a poorer digestate's dewaterability (De la Rubia et al. 2013). Thus, there is a great interest to search for alternative technologies.

The enzymatic hydrolysis of WAS is commonly described by empirical first-order kinetics (Vavilin et al., 2008), meaning that the observed solids conversion rate is dependant on the solid substrate concentration and the first-order hydrolysis rate constant (Eq. (1)).

$$\frac{dS}{dt} = -k_H S \tag{1}$$

Where S = substrate concentration, t = time, and k_H = first-order hydrolysis rate constant.

Theoretically, in a CSTR, the concentration S in the reactor equals the effluent S concentration, indicating that in-reactor conversion rates decrease with decreasing S (Eq. (1)), agreeing with an increased conversion ratio (η) (Fig. 1a). Based on Eq. 2, the required volume of a CSTR at a given inlet feeding rate (F_0) is fully determined by the required η and is graphically presented by the large rectangular area shown in Fig. 1b (Levenspiel 2006). On the contrary, by cascading CSTRs, small reactor volumes in series are applied that result in high intermediate S concentrations. Consequently, the first CSTRs can be operated at high reaction rates, whereas the last CSTR of the cascade system will have a similar reaction rate as the single stage CSTR. Thus, the series of small CSTRs will eventually reach to a similar η but to a significant smaller working volume, compared to the single stage CSTR (Fig. 1c). The overall required volume of the cascade system is reciprocally correlated to the number of CSTRs.

$$V = F_0 \frac{1}{-r} \eta \tag{2}$$

Where V = volume of the CSTR (m^3), F_0 = substrate feeding rate (kg COD/day), $-r$ = substrate conversion rate (kg COD/m^3/day), and η = substrate conversion ratio (0–100%).

Cascade CSTR configurations are commonly applied to accelerate catalytic substrate conversions that are characterised by Eq. (1) (Miyawaki et al., 2016). In case reaction rates are substrate dependant, such as for soluble substrates in Michaelis-Menten and/or Monod kinetics, the impact of reactor cascading will even be higher. However, for solid substrates such as WAS, concentration dependant reaction rates are rarely documented (Miron et al., 2000) and generally first-order reaction rate constants are considered (Blumensaat and Keller 2005).

Up to now, application of the cascade CSTR configurations for WAS has been mainly reported in the scope of co-digestion in food waste (Liu et al., 2013) or agricultural waste (Zhou et al., 2019), in which WAS contributed to improved buffer capacities and more balanced nutrient profiles. In the past decade, several researchers found higher WAS conversion efficiencies by using two-stage (two CSTRs in series) mesophilic AD systems, either with or without addition of primary sludge, for which no clear mechanistic explanation was given (Athanasoulia et al., 2012; Maspolim et al., 2015b). Ge et al. (2011b) and Wu et al. (2015) observed an improved hydrolysis rate in temperature-phased (thermophilic CSTR–mesophilic CSTR) WAS anaerobic digestion processes. Nonetheless, the authors attributed the enhanced hydrolysis merely to the thermophilic conditions applied. Despite the fact that staging has resulted in improved WAS digestion, it remains unclear whether accelerated enzyme activities, increased surface area of the solid substrates, and/or other factors were determinative. However, the published wide range of assessed hydrolysis rate constants for WAS (Batstone et al., 2002), gives room for further research and process optimisation.

In order to (1) comprehensively clarify whether a cascade configuration enhances WAS hydrolysis, and to (2) better understand the governing hydrolysis kinetics in this system, a novel cascade AD system for WAS treatment was researched in this study, which consisted of four CSTRs in series, i.e., three small-volume CSTRs and a large-volume CSTR. Considering that digestate recycle improves process stability in staged anaerobic digestion (Qin et al., 2019), the cascade system was equipped with a modest digestate recirculation, applying a much lower ratio than reported in literature (Wu et al., 2015). As such, the whole system can be interpreted as a semi plug-flow device with only a negligible hydraulic impact of the recycle flow. Reactor performance in the different steps of the system were investigated. Detailed research on prevailing specific hydrolysis rates, activities of key hydrolytic enzymes and the bacterial/archaeal community structure was performed to explain the results of the reactor performance and unveil the impact of cascading on hydrolysis kinetics. All results from the cascade system were compared to those obtained from a reference conventional CSTR system operated under the same conditions with regard to feeding regime, total organic loading and temperature.
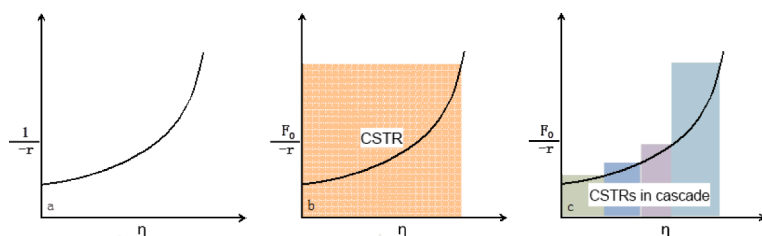
Fig. 1. Theoretical reactor volume reduction by applying a cascade CSTR configuration versus a single stage CSTR (Levenspiel 2006). (a) Relationship between the substrate conversion ratio (η) and the reciprocal first order conversion reaction rate (-r), (b) required reactor volume of a single stage CSTR for a required conversion ratio η, indicated by the total surface area of the rectangle, and (c) required reactor volume for the cascade CSTR configuration for the same η, by summing the 4 subsequent surface areas.

Fig. 2. Schematic illustration of the experimental set-ups.

Table 1
Operational conditions of the cascade AD system and the reference-CSTR.

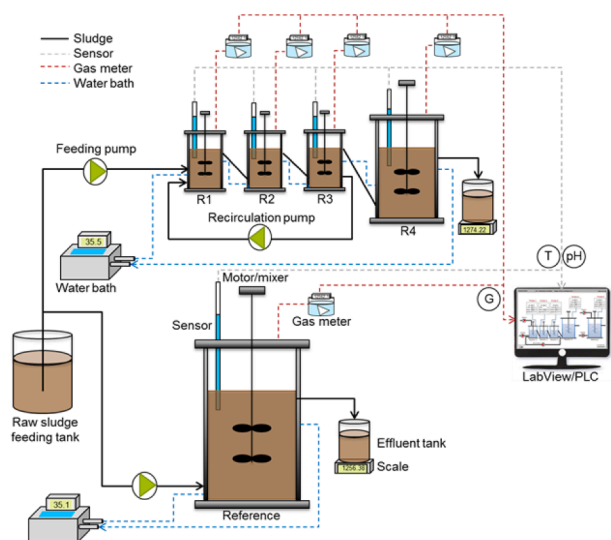| Experimental time (day)	SRT (days) Cascade System	Reference-CSTR	Total organic loading rate (g COD/L/d)	Phase
0–71	R1-R3: 2.2 each R4: 15.4	Reference: 22	2.41	I
72–152	R1-R3: 2.2 each R4: 15.4	Reference: 22	2.41	II
153–259	R1-R3: 1.5 each R4: 10.5	Reference: 15	3.54	III
260–330	R1-R3: 1.2 each R4: 8.4	Reference: 12	4.41	IV

Materials and methods

Source and characteristics of inoculum and substrate

All reactors were seeded with anaerobic sludge collected from a full-scale mesophilic anaerobic digester (SRT of 20 days) at the municipal WWTP Harnaschpolder, The Netherlands, treating primary sludge and centrifuge-thickened WAS from an enhanced biological phosphorus removal (EBPR) process. More information regarding the configuration and the operational parameters of the EBPR process can be found elsewhere (Guo et al., 2020b). The inoculum characteristics were: pH 8.1 ± 0.4, total solids (TS) 3.3 ± 0.1 wt%, and volatile solids (VS) 2.3 ± 0.0 wt%. The WAS from the same WWTP was collected weekly as feed sludge, and was characterized by a total chemical oxygen demand (tCOD) concentration between 40 and 70 g/L. The tCOD concentration of the feed sludge was adjusted to approximately 53 g/L by centrifugation or dilution with the fresh centrifuged supernatant obtained from the same WWTP, and stored at 4 °C before use.

Experimental set-up and operation

The experiments were carried out using two digestion systems operated in parallel: 1) a cascade AD system consisting of three CSTRs with 2.2 L each (R1, R2, R3) and a 15.4 L CSTR (R4); 2) a conventional CSTR as the reference with a working volume of 22 L (Fig. 2). The experimental set-ups were both equipped with feed pumps (Watson-Marlow 120 U/DV-220Du, USA), temperature & pH sensors (Endress & Hauser, The Netherlands), and biogas flow meters (Ritter Milligas Counter MGC-1-PMMA, Germany). The digestate was discharged from all reactors in both systems via overflow. In addition, for the cascade system a sludge recirculation system from R3 to R1 with a flow ratio of 10% (recirculation/feed) was implemented using a recirculation pump (Watson-Marlow 120 U/DV-220Du, USA). The temperature of all water-jacket equipped CSTRs was 35 ± 1 °C, controlled by thermostatic water baths (Tamson Instruments, The Netherlands). Both systems were

monitored via a computer running LabView software (National Instruments, USA).

The total SRT of both systems was decreased from 22 to 12 days in four phases. The operational conditions during all these phases are shown in Table 1.

Analysis and calculation methods

The tCOD and soluble COD (sCOD) were measured using spectrophotometry-based test kits (Hach Lange LCK, Germany). TS and VS were analysed according to standard protocols (APHA, 2005). The pH was determined with a multi-functional metre (WTW Multi 720, Germany). VFAs were measured by a gas chromatograph (GC) equipped with a flame ionisation detector (FID) (Agilent 7890A, USA) and a column (Agilent 19091F-112). Helium was used as carrier gas (1.8 mL/min); injection port and oven temperatures were 240 °C and 80 °C, respectively. Methane content of the biogas was analysed using a GC (Varian CP 4900, USA) with thermal conductivity detector (TCD) and columns, i.e. Mol-Sieve-5A-PLOT and argon as carrier gas (1.47 mL/min, 80 °C) and PoraPlot-U and helium as carrier gas (1.47 mL/min, 65 °C).

The specific hydrolysis rate, referring to the hydrolysis rate constant k_H (Yasui et al., 2008), was calculated by Eq. (3) based on Wu et al. (2015). As the AD system was equipped with a digestate recirculation of 10% from R3 to R1, the recycled sCOD and the recycle flow were also considered in the calculation of the specific hydrolysis rates for these three reactors.

$$\text{Specific hydrolysis rate (g COD / g VS / day or 1 / day)} = \frac{\left(\frac{\text{mass\_sCOD+mass\_COD}_{CH_4}}{\text{day}}\right)_{\text{eff.}} - \left(\frac{\text{mass\_sCOD}}{\text{day}}\right)_{\text{inf.}}}{\text{mass of VS within reactor}} \tag{3}$$

Where mass_sCOD = sCOD weight (g); mass_COD$_{CH_4}$ = CH$_4$wt calculated as COD (g); eff. = effluent + methane; inf. = influent. It should be noted that inf. for R1 is composed of both the feeding and the recirculated digestate; inf. for R2 is the effluent from R1; inf. for R3 is the effluent from R2; inf. for R4 is composed of the effluent from R3, without the recycle flow.

Hydrolytic enzyme activity

Sampling and enzyme extraction

Triplicate sludge samples, including feed and digestates, were collected for enzyme extraction at the end of Phase-II (day 145 and 151),

Phase-III (day 252 and 258) and Phase-IV (323 and 329) of the individual reactors of both digestion systems. The hydrolytic enzymes were separated into free and sludge-attached fractions. The free enzymes are defined as the enzymes that are present in the WAS's supernatant, whereas the sludge-attached enzymes are either membrane-bound or in other ways attached to the sludge particles. The extraction method of the hydrolytic enzymes was implemented according to Zhang et al. (2007) with a slight modification for sludge samples. Briefly, 1 mL sludge sample was centrifuged in a 1.5-mL tube (Eppendorf, Germany) at 14,000 rpm for 1 min. The supernatant was transferred to a clean tube and was used for the measurement of free enzyme activities. The pellet was washed twice, using potassium dihydrogen phosphate buffer (pH 7.0, 0.1 mol/L) and was subsequently resuspended in sodium acetate buffer at pH 6.0 to the original volume to release sludge-attached enzymes. After centrifugation the suspension at 3000 g for 10 min, the supernatant was used for the determination of sludge-attached enzyme activities.

Quantification of enzyme activity

This work mainly focused on two hydrolytic enzymes: protease and cellulase. The activities of protease and cellulase were individually analysed by Pierce fluorescent protease assay kit (Thermo Fisher, USA) and MarkerGene fluorescent cellulase assay kit (MarkerGene, USA), using a 96-well microplate spectrophotometer (BioTek Synergy-HTX, USA). Meanwhile, API ZYM® strip (BioMerieux, France) was used to determine the activities of specific amino peptidases (leucine arylamidase, valine arylamidase and cystine arylamidase) and glycosyl-hydrolases (α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, n-acetyl glucosamidase, α-mannosidase and α-fucosidase). This commercial semi-quantitative micro-cell method works via colour development, with a numerical level of 1–5 (from low, 5 nmol, to high, >20 nmol) assigned to each sample, based on the colour chart provided by the manufacturer. The measurements of enzyme activity for both methods were performed at 35 °C.

Microbial community analysis

During the experiment, duplicate biomass samples were analysed to evaluate the microbial community dynamics, including one inoculum sample, two feed samples and 15 digestate samples from the digestion systems. The feed samples were taken individually on day 79 (summer season) and day 235 (winter season), and the digestates were sampled from R1, R2, R3, R4 and reference-CSTR at the end of each phase, i.e. days 151, 258, and 329. The FastDNA® SPIN-Kit-for-Soil (MP Biomedicals, USA) was used to extract DNA according to the manufacturer's instructions. The obtained DNA's quality was checked by Qubit3.0 DNA detection (Qubit® dsDNA-HS-Assay-Kit, Life Technologies, USA). High throughput sequencing was performed using the HiSeq Illumina platform and a universal primer 515F/806R (5′-GTGCCAGCMGCCGCGGTAA-3′/5′-GGACTACHVGGGTWTCTAAT-3′) for bacterial and archaeal 16S rRNA genes (Novogene, UK). Raw reads were deposited in the European Nucleotide Archive under accession number PRJEB40450. Sequences were analysed by the QIIME pipelines (Version 1.7.0) to pair forward and reverse sequences, and removal of chimeras' sequences was performed by UCHIME algorithm.

Sequences with ≥ 97% similarity were clustered into one operational taxonomic unit (OTUs) by UCLUST algorithm. Singletons were removed, and OTUs with an occurrence less than three times in at least one sample were excluded. Taxonomic assignment was performed in Mothur software against the SILVA Database.

Statistical analysis

Student's *t*-test was used for variance analysis by SPSS Statistics 25 (IBM, USA), with the threshold for significance set at a P-value < 0.05. Shannon index and principal coordinate analysis (PCoA) based on the ordination of Bray-Curtis similarities were used to evaluate Alpha diversity and Beta diversity, respectively, by "vegan" microbial community ecology package in R software (version 4.0.2). Prediction of functional pathways from 16S rRNA gene sequences were conducted by
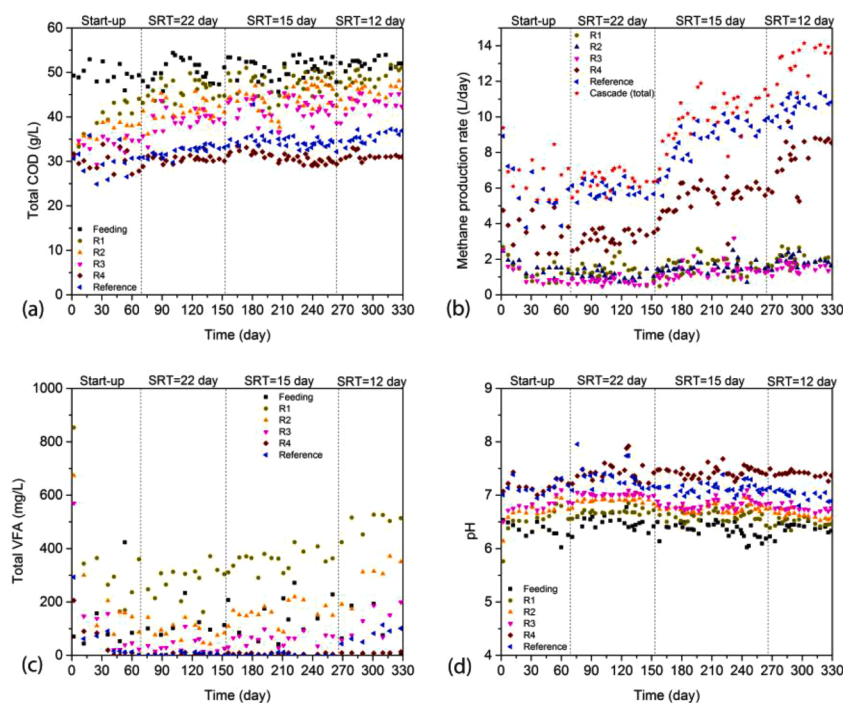

Fig. 3. Operational performance of the cascade AD system and the reference digester, respectively. (a) tCOD concentration, (b) methane production rate, (c) total VFA concentration and (d) pH.

"Tax4Fun2" software package that provides functional annotations based on the Kyoto encyclopedia of Genes and Genomes (KEGG) pathway database.

Results and discussion

Performance comparison between the cascade AD system and the reference-CSTR

During the start-up phase, the effluent tCOD concentrations and methane production rate fluctuated in all reactors (Fig. 3a). Both parameters gradually stabilised from day 71 onward, after which the cascade and reference system were both operated under stable conditions for 81 days (Phase-II). During both Phase-I and Phase-II, the cascade system and the reference-CSTR were operated with an SRT of 22 days. The tCOD removal efficiency of the entire cascade system was 43 ± 6%, versus 40 ± 5% of the reference-CSTR during this period. Both removal efficiencies were within typical ranges of mesophilic WAS digestion, reported by previous studies (Maspolim et al., 2015b). On average, the methane production rate was around 8% higher in the cascade system than in the reference-CSTR (Fig. 3b).

After the total SRT was lowered to 15 days (Phase-III), effluent tCOD concentrations of both R4 and the reference-CSTR increased due to the sudden increase in total organic loading rate (OLR) from 2.4 to 3.5 g COD/L/d. This reduction in tCOD removal efficiency was also observed at the start of Phase-IV, when the total SRT was further decreased to 12 days and the total OLR correspondingly increased to 4.4 g COD/L/d. Strikingly, only the cascade system recovered to a tCOD removal efficiency between 40% and 42% at the applied increased OLR, whereas the tCOD removal efficiency in the reference-CSTR reduced to around 38% in Phase-III and 31% in Phase-IV. The difference in treatment performance was reflected by the increasing difference in methane production (Fig. 3b). The cascade system showed an average 13% higher methane production rate in Phase-III and even an average 29% higher rate in Phase-IV than the reference-CSTR. The obtained results clearly demonstrated the advantage of applying a cascade configuration, particularly at reduced SRTs. In fact, at 12 days SRT, the overall capacity referring to the tCOD removal efficiency of the cascade digester was 30–35% higher, compared to the reference-CSTR with the same total volume.

Zooming into the separate reactors of the cascade system reveals that the reactors R1, R2 and R3, with an SRT of 2.2 days each (Phase-II), contributed to 20–24% of the total methane volume that was produced in the cascade system (Fig. 3b). These results agree with a reported study on two staged AD systems under similar SRT conditions, which showed that the methane production in the first CSTR was on average 25% of the total (Maspolim et al., 2015b). When the SRT in the cascade reactors R1, R2 and R3 was decreased to 1.5 and 1.2 days each in Phase-III and Phase-IV, respectively, the methane production stayed between 12 and 16% of the overall total methane production. The biogas in these three reactors contained 46–53% methane, while the methane content of the biogas of R4 and of the reference-CSTR was 56–62%. Negligible hydrogen partial pressure was found in all the anaerobic reactors (< 0.01%). These observations showed that, despite their short SRT and most probably due to the 10% recirculation flow, active methanogens were present in R1, R2 and R3.

VFA concentrations and pH are commonly used as indicators for process perturbation and/or reactor control (Franke-Whittle et al., 2014). The total VFA concentration in the feed and all reactors is presented in Fig. 3c. As expected, the VFA concentration was always the highest in R1 and was gradually reduced along the system. Acetate and propionate accounted for 60–80% of the total VFAs, showing their predominance in all reactors (Fig. S1 in supplementary materials). With increased OLR, or decreased SRT, an elevation in VFA concentration in R1, R2 and R3 was observed, from 310, 100 and 60 mg/L at SRT 22 days to 590, 380 and 175 mg/L at SRT 12 days, respectively. Very low total VFAs (< 5 mg/L) were found in reactor R4 in all phases, demonstrating

that all VFAs were eventually converted to methane in the last step of the cascade system. In the reference-CSTR there was no VFA accumulation observed, even at the shortest SRT (12 days) when total VFA concentration slightly increased to around 110 mg/L. Clearly, the VFA concentrations remained far below the inhibition threshold for methanogenic activity (Wang et al., 2009), and thus cannot explain the difference in WAS degradation between the cascade system and the reference-CSTR at short SRTs. However, the pH in both R1 and R2 of the cascade system was between 6.3 and 6.5, somewhat lower than the pH in the rest of the reactors. The lower pH coincided with the somewhat higher VFA concentrations in R1 and R2 and can be attributed to increased acidifying activity and reduced methanogenic activity in the first reactors of the cascade (Maspolim et al., 2015b). In reactors R3 and R4, as well as in the reference system, the pH remained neutral (Fig. 3d). Nonetheless, the relatively stable pH in R1 and R2 could be ascribed to alkalinity supplementation by digestate recirculation from R3 to R1, introducing sufficient buffer capacity as presented in Fig. S2 in Supplementary material.

To be able to explain the different tCOD removal efficiencies between the cascade and the reference system, the specific hydrolysis rates were calculated using Eq. (3), the tCOD and sCOD variations (Fig. 1 and Fig. S3 in Supplementary materials), and the methane production (Fig. 3b) in each reactor. Computed specific hydrolysis rates, resembling the first-order hydrolysis rate constant k_H (Eq. (1)), are shown in Fig. 4. Under all tested operational conditions, the specific hydrolysis rate was highest in R1 of the cascade system, and steadily decreased throughout the subsequent reactors of the cascade. During Phase-II, the specific hydrolysis rate calculated for the reference-CSTR was slightly higher than that in R3 of the cascade system. Reducing the SRT from 22 to 12 days led to approximately a doubling of the specific hydrolysis rate in the reactors of the cascade system, while it increased only 1.5 times in the reference-CSTR. It should be noted that the bar-presented specific hydrolysis rates are in fact underestimates of the actual values, since these were calculated using Eq. (3), which includes both the substrate and biomass VS in each reactor. However, particularly in reactors R1–3, the contribution of the substrate VS to the total VS is relatively large. We, therefore, recalculated the apparent k_H values using the VS content in R4, which resembles the non-digestible VS fraction in the entire cascade system. The corrected k_H values are presented above each bar of R1–3 in Fig. 4, showing an even higher increase in specific hydrolysis rates in the first stages of the cascade reactor.

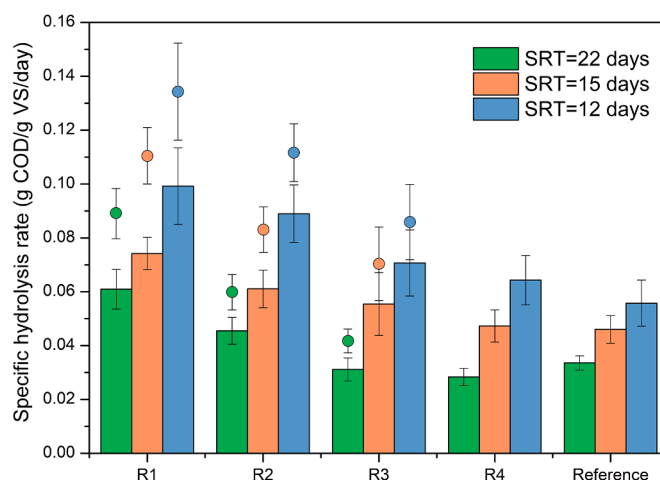Strikingly, under all loading conditions, the assessed specific

Fig. 4. Specific hydrolysis rate calculated based on the COD balance and the VS concentration of each reactor (bars). The circles displayed above the bars of R1, R2 and R3 represent the calculated specific hydrolysis rate using the stabilized VS concentration of R4 at each SRT, which resembles the non-digestible VS fraction in the cascade system.

hydrolysis rates in R4 of the cascade system and the reference-CSTR were very similar. Nonetheless, at the highest OLR, the overall specific hydrolysis rate in the reference-CSTR was significantly lower (p-value < 0.05) than the separate specific hydrolysis rates in all reactors of the cascade system. Apparently, the specific hydrolysis rate was process-condition dependant and results in Fig. 4 showed that in all reactors the specific hydrolysis rate increased with increasing OLRs. Similar observation were previously done by Miron et al. (2000). Our present results clearly indicate that the potential volume reduction, which can be attained by implementing cascade configurations, is indeed much more than based on solely the theoretical considerations as explained in Fig. 1 (Levenspiel 2006), where the same first-order reaction rate is applied for all individual reactors in the cascade system and the single stage CSTR. Moreover, at the applied low SRTs, or imposed extreme OLRs, the specific hydrolysis rates increased significantly more (p-value < 0.05) in the first reactors of the cascade system compared to that in the reference-CSTR (Fig 4). Most likely, the maximum organic loading potentials of the cascade system were not reached yet, as process performance remained stable even at an SRT of 12 days (Fig. 3).

Results further indicate that for increasing the sludge treatment capacity at a common WWTP, the present AD installation can be upgraded in a relatively easy manner to a very compact cascade reactor system via retrofitting existing parallel-fed large-scale conventional CSTR-based sludge digesters. For instance, one CSTR digester could be divided into a sequence of several compartments and subsequently be connected with another digester in series.

Hydrolytic enzyme activity

To explain the large differences in observed specific hydrolysis rates between the different reactors, the hydrolytic enzyme activities were assessed (Parawira et al., 2005). Cellulosic fibres and proteins are identified as the two predominated organic components in WAS (Guo et al., 2020b). Therefore, the activity of cellulase and protease were chosen as representative enzyme activities for a first characterisation of WAS hydrolysis in both systems, applying a widely reported enzymes extraction protocol for anaerobic samples (Zhang et al., 2007). Meanwhile, automatic measurements in a 96-well microplate reader rather than manual measurements were conducted for the analysis of enzyme activities in this study (Bonilla et al., 2018), with the duplicate extraction of enzymes from the same reactor at three inconsecutive days. Results in Fig. 5 showed that both free and sludge-attached enzymes are present in the digester, regardless of the configuration type, i.e. cascade or single CSTR. The results showed that protease activities were two orders of magnitude higher than cellulase activities, which could be possibly due to the significant higher proportion of protein than

cellulose in WAS (Guo et al., 2020b). Highest protease and cellulase enzyme activities were present in the sludge-attached fraction of both reactor configurations. Enzyme activities are proportionally related to the enzyme's amount (Kim et al., 2012), suggesting that the hydrolytic enzymes were mainly adsorbed on, or attached to the sludge matrix, in line with a previous publication by Maspolim et al. (2015a).

In both free and sludge-attached fractions, the activity of hydrolytic enzymes distinctly increased from the feed to R1, especially at short SRTs, indicating that hydrolysis in R1 was indeed accelerated owing to increased presence of hydrolytic enzymes. Significant higher enzyme activities (p-value < 0.05) were observed in the three small reactors in comparison with the reference-CSTR: the protease activities in R1 were double the activities in the reference-CSTR; even the protease activities in R4 were slightly higher than those in the reference-CSTR. Meanwhile, the cellulase activities in R1, R2 and R3 were statistically higher than those in R4, while the digestate of R4 showed a similar cellulase activity as the reference-CSTR (Fig. 5d). The observed higher hydrolytic enzyme activities in the cascade AD system, compared to those of the reference-CSTR, could be attributed to the imposed high OLRs (corresponding to short SRTs) in reactors R1, R2 and R3. Following first order reaction kinetics (Eq. (1)), the application of increased OLRs results in accelerated hydrolytic enzyme activities (Menzel et al., 2020; Xiao et al., 2017). Results showed that enzyme activities, especially the sludge-attached ones, in all reactors increased over three times when the total SRT was reduced from 22 to 12 days (Fig. 5). Notably, the increase in the enzyme activities in both systems exceeded the increase in the calculated specific hydrolysis rates in each reactor (Fig. 4). This mismatch strongly indicates that the actual solids hydrolysis in the cascade system was limited by the available free surface for enzymatic attack, rather than by the presence of sufficient hydrolytic conversion capacity.

A more detailed semi-quantitative analysis of amino peptidases and glycosyl-hydrolases in both free and sludge-attached fractions using API ZYM® strips, were carried out at the same moments as described above (Fig. 6). Similar to protease and cellulase activities, the activities of all hydrolases tested with this method increased at short SRTs, and showed a downward trend in activity from R1 to R4 of the cascade digester. Surprisingly, however, the β-glucuronidase, α-mannosidase and α-fucosidase activities increased stepwise along the cascade system, which indicates that the hydrolysis of target substrates of these enzymes occurs later in the process. The presence and the role of the target substrates, namely, glucuronic acid, mannose and fucose in the sludge matrix have been researched in several studies, showing that they act as main building blocks in the structural extracellular polymeric substances (SEPS) that form the gel-like structures of the sludge (Guo et al., 2020a). Regarding the degradation of SEPS in both digestion systems (Fig. S4 in Supplementary materials), results showed that SEPS were mostly
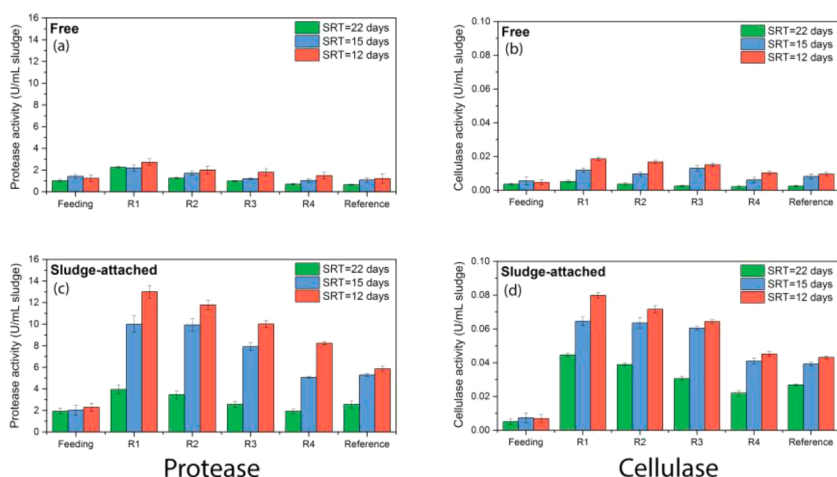

Fig. 5. Enzyme activity in U per mL of sludge from the feeding and each reactor. The TS concentration of the feeding was: 4.1–4.2 wt%, and of each individual reactor: 3.9–4.1, 3.7–4.0, 3.0–3.7, 2.8–3.0 and 3.1–3.3 wt% for R1, R2, R3, R4 and the reference-CSTR, respectively. (a) Free protease. (b) Free cellulase. (c) Sludge-attached protease. (d) Sludge-attached cellulase. Samples for each measurement were taken during stabilised performance at each operational period. Error bars refer to the standard deviation ($n = 6$).
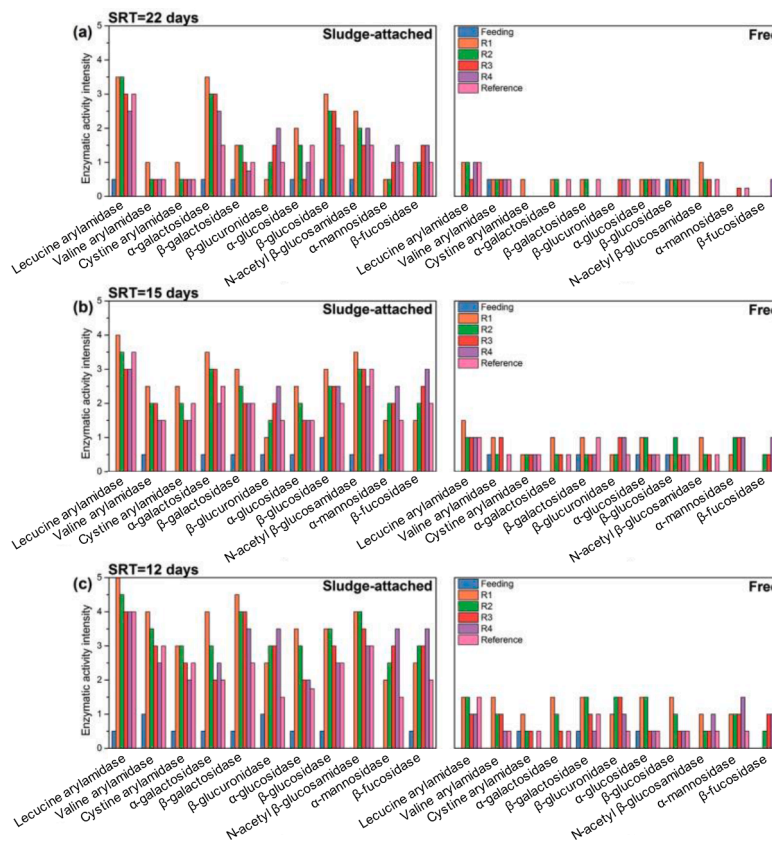
Protease   Cellulase

Fig. 6. Average (*n* = 3) enzyme intensity of specific amino peptidases and glycosyl-hydrolases in both cascade system and reference-CSTR, which were analysed using API ZYM® strips for sludge-attached and free enzymes collected at the end of (a) SRT = 22 days, (b) SRT = 15 days and (c) SRT = 12 days.

converted in R4, irrespective of changes in SRT, which was in line with the distributions of the β-glucuronidase, α-mannosidase and α-fucosidase activities. In addition, observations from the cascade system reveal that in the first reactors the more easily biodegradable (poly-)saccharides and (poly-)proteins were degraded, while in the remaining of the cascade system, the more refractory organic residuals in WAS, such as SEPS related saccharides, were degraded. As a consequence, the cascade system revealed a more stepwise and improved reduction of different types of organics, which e.g. resulted in 14% more SEPS reduction at the total SRT of 22 days compared to the reference-CSTR. At the shortest tested SRT of 12 days, SEPS reduction was even 64% higher (Fig. S4 in Supplementary materials).

Pyrosequencing analysis of the microbial communities

Diversity indices

The results of Alpha diversity based on Shannon diversity were listed in Table S in Supplementary material. Substrate sample 1 & 2, and the inoculum had the highest and lowest values, respectively, meaning that the WAS substrate contained the most diverse bacterial communities, whereas the anaerobically grown inoculum had the least biodiversity. Shannon diversity decreased in both AD systems when operated at the total SRT of 22 days and slightly increased as the SRT was reduced. This indicates that the initial microbiome members that were present in the feed partially disappeared in the cascade AD process and thus, a narrowed AD community was eventually formed.

A microbial dynamic transition alongside with the cascade system from R1 to R4 could be clearly demonstrated by the Beta diversity described via PCoA based on the matrix distance between the samples (Fig. 7). In all operational conditions, R1, R2 and R3 were clustering closely to each other, while R4 was obviously separated from R1–3 and near the inoculum, revealing a different microbial composition presented in R4 compared to other reactors in the cascade system. The microbial structure of the reference-CSTR and the R1–3 was similar to that of WAS under the reduced SRTs, suggesting less cell decay of the fed WAS at this short SRT, which is possibly linked to the deterioration in tCOD reduction efficiency (Fig. 3a).

It should be noted that the applied cascade AD system was equipped with a digestate recirculation system operating at a recirculation ratio of 10%. It has been reported that recycling the digestate from a
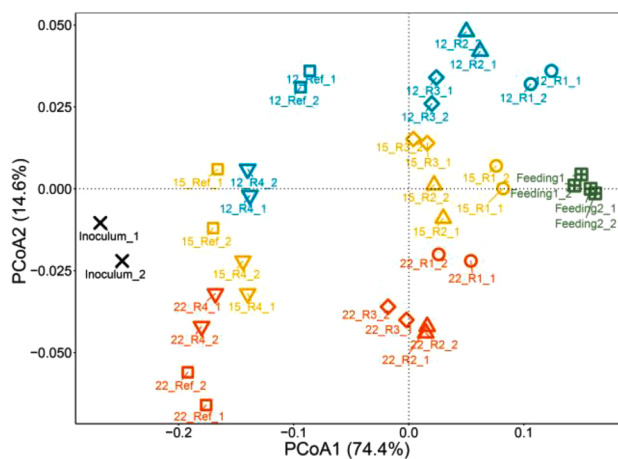

Fig. 7. PCoA analysis for the microbial community of feed sludge, inoculum and digestates from all reactors operated at the different SRTs. The samples analysed for PCoA were plotted in duplicates grouped by the same symbol and colour. Symbols in black and green represented the inoculum and the feed, respectively. Symbols in blue, yellow and red refer to the reactors operated at SRT of 12 days, 15 days and 22 days, respectively.
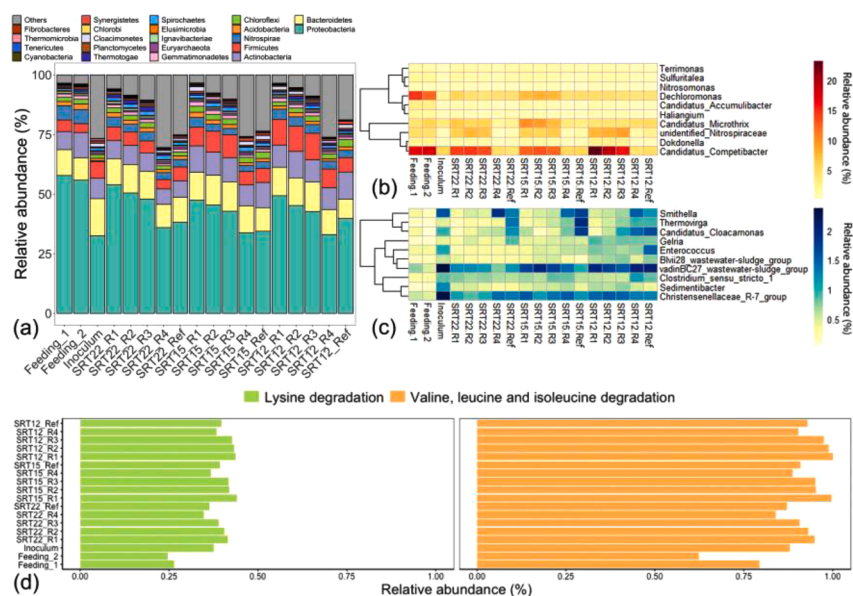
Fig. 8. (a) Species taxonomy of bacteria at the phylum level: the species, whose sums of percentage in all the samples are less than 1%, are classified as "the others", (b) relative abundance of taxa related to feeding dominated bacteria of interest (top 10 species that presented in feeding), (c) anaerobic hydrolytic bacteria (top 10 species that appeared in all anaerobic reactors) at the genus level and (d) prediction of functional pathways related to amino acids degradation (average value, $n = 2$).

methanogenic reactor to an acidogenic reactor at a recycling ratio of 100% resulted in a changed and improved diversity of bacteria and archaea in the acidogenic reactor (Wu et al., 2016). Thus far, the effect of only 10% recycling is unknown. Nonetheless, in our present study, considerable methane production was observed in R1, R2 and R3 (Fig. 3b), which might be ascribed to the supplement of methanogens via digestate recirculation. However, based on the PCoA results, showing the clear microbial shift within the cascade system from R1 to R4 (Fig. 7), it seems that this impact of 10% recycling was limited.

Bacterial communities

The bacterial species taxonomy at phylum level is shown in Fig. 8a. Proteobacteria (55–60%), followed by Bacteroidetes (8–10%) and Actinobacteria (7–9%) were the most dominant phyla in the raw WAS, which is in line with previous studies (Westerholm et al., 2016). The changes in microbial composition between samples were most pronounced for Proteobacteria, because the total reduction in the relative abundance of this phylum was distinctly higher than for the other phyla in both cascade system and reference-CSTR. The relative abundance of the genus *Candidatus_Competibacter* belonging to the phylum Proteobacteria was reduced by approximately 30% in R1, R2 and R3 together, while it was declined by 60% in the post digester (R4) of the cascade AD system. A similar observation was also found for other genera from this phylum, such as *Candidatus_Accumulibacter* related to phosphorus removal and *Dechloromonas* sp. for denitrification (Luo et al., 2020), even though the fractions in WAS were relatively low in this study (Fig. 8b). The results imply that the aforementioned dominant phyla largely disappeared due to cell decay in the AD process. Considering that 9–24% of WAS consists of microorganisms (Gonzalez et al., 2018), the released amount of intracellular organics due to endogenous decay of cells cannot be ignored in the cascade system and would become part of the tCOD that was available as substrate for the investigated hydrolytic enzymes (Fig. 5 and 6). Firmicutes were not predominant in the WAS, but clearly, the relative abundance of this phylum increased in R1, R2 and R3 to approximately 6.7% compared to 4.5% in the feed sludge at the total SRT of 22 days, Furthermore, the relative abundance of Firmicutes increased to 8.8% and 11.3% as the SRT reduced to 15 and 12 days, respectively. Firmicutes have been identified to hydrolyse and ferment large numbers of organic compounds under a variety of conditions in AD systems (Karthikeyan et al., 2016; Liu et al., 2019). The

increase in relative abundance of this type of species implies that the role of hydrolysis and acidogenesis processes in R1, R2 and R3 of the cascade system became increasingly more important as the SRTs decreased, in line as was reported by Zhang et al. (2019).

To relate the identified microbes to hydrolysis and acidogenesis of WAS in the cascade AD system under different operational conditions, the top 10 genera that governed the hydrolysis/acidogenesis of the organic compounds in both systems were selected and ranked by the relative abundance, while the changes in relative abundance were shown in Fig. 8c. Bacteria affiliated to genera *VadinBC27_wastewater-sludge_group, Clostridium_sensu_stricto_1, Enterococcus, Gelria, Bivil28_wastewater-sludge_group* and *Sedimentibacter* had significantly higher (p-value < 0.05) relative abundance in R1, R2 and R3 than in the reference-CSTR at the SRT of 22 days. This might have been due to the greater abundance of non-hydrolysed substrates that were present in R1, R2, and R3, since these genera have been frequently reported as the prevalent fermenters that were capable of hydrolysing protein or carbohydrate in AD (Kirkegaard et al., 2017; Liu et al., 2016; Wang et al., 2020). Possibly, the mentioned genera can be recognised as the main contributors to the enhanced hydrolysis rate in the cascade digester system. Moreover, lowering the total SRT of these reactors further increased the relative abundance of the aforementioned bacteria, which implies their higher metabolic activities in the degradation of WAS at higher loading rates. On the other hand, an upward trend in relative abundance of *Smithella, Candidatus_Cloacamonas* and *Thermovirga* was detected in the cascade system, especially in R1, R2 and R3, at the short SRTs. These recently characterized microorganisms might oxidize propionate and ferment sugars and amino acids to produce hydrogen and carbon dioxide, indicating that these species may possibly constitute acidifying and syntrophic associations (Stolze et al., 2015; Zamanzadeh et al., 2013). It should be noted that a small proportion of *VadinBC27_wastewater-sludge_group* and *Bivil28_wastewater-sludge_group* was also found in WAS. These strains directly might have contributed to the hydrolysis and degradation of WAS when they entered the cascade system. Moreover, because of their continuous seeding, they might have persisted as functional biomass in R1–3, where short SRTs were applied (Kim and Speece 2002). However, the exact role of the WAS-related cultures in the cascade system needs further studies.

Besides the investigation on the relative abundance of the functional bacteria, the microbial functional pathways including amino acid and

carbohydrates metabolisms in different experimental phases were also researched and the results were summarized in the Excel file, Supplementary materials. It was found that lysine degradation (Ko00310) as well as valine, leucine and isoleucine degradation (Ko00280) were the dominant pathways related to biomass conversion. The relative abundance of these metabolic pathways in the different reactors indeed increased when the reactor SRT dropped from 22 to 12 days. Moreover, they showed a similar trend as the activities of valine and leucine arylamidase that catalyse the hydrolysis of valine and leucine from peptide chains (Fig. 8d and Fig. 6). These findings suggest that applying a cascade system results in an enhanced microbial metabolism of hydrolytic/acidogenic bacteria that caused the observed acceleration in hydrolytic enzyme activity and subsequent enhanced sludge reduction compared to the reference-CSTR. Obtained results also illustrated the microbial complexity of WAS hydrolysis, which is difficult to capture in first-order hydrolysis kinetics, particularly under high loading conditions.

Methanogenic archaeal communities

As for the archaeal domain displayed in Fig. 9, *Methanobrevibacter* and *Methanosaeta* were equally dominant in the feed (around 28% each in relative abundance). In Phase-II, when the cascade system was operated at an SRT of 22 days, *Methanobrevibacter*, a hydrogenotrophic methanogen, was the most abundant methanogen in R1, but gradually became the minor species in favour of *Methanosaeta* that utilize acetate as the sole substrate from R2 to R4 (Maspolim et al., 2015c). This means a clear microbial shift from hydrogenotrophic methanogens towards acetoclastic methanogens alongside the cascade system. Also, at the low SRTs of 15 and 12 days in Phase-III and Phase-IV, the composition of methanogens in R1–4 followed a similar trend as in Phase-II, whereas the proportion of hydrogenotrophic methanogens was at a higher level. The predominance and importance of hydrogenotrophic methanogens in the acidogenic first stage reactors of phased AD systems have been described in literature. The very high OLR in the first stages of the cascade system resulted in a very high acidogenesis rate, with a concomitant high hydrogen and carbon dioxide production rate (Huang et al., 2015; Liu et al., 2019; Shimada et al., 2011; Zhang et al., 2019). The increased hydrogen/carbon dioxide flux in reactors R1–3 resulted in an increased yield of hydrogenotrophic methanogens, leading to an increase and eventual dominance of hydrogenotrophic methanogenic subpopulations. Considering the maximum growth rate (μ_{max}) of hydrogenotrophic methanogens in the range between 2.00–2.85 d^{-1}, which is around 3–9 folds that of *Methanosaeta* species (0.33–0.71 d^{-1}) (Batstone et al., 2002; Van Lier et al. 2020), and the very short SRTs of reactors R1–3, the microbial abundancy of hydrogenotrophic methanogens could outcompete the acetoclastic *Methanosaeta* species. In contrast, the applied SRT in R4 and the prevailing acetate flux resulted in a pre-dominance of *Methanosaeta* in the final reactor of the cascade. Notably, the acetate concentrations in R1, R2 and R3 were all significantly higher (p-value < 0.05) than the threshold (< 600 µg/L for *Methanosaeta* survival (Klocke et al., 2008). Therefore, unlike the reference-CSTR and R4, methane production from hydrogen and carbon dioxide, rather than from acetate, was most likely the dominating methanogenic pathway in R1, R2 and R3.

It is noteworthy that sequences affiliated with *Methanospirillum* had promoted relative abundance in all digesters from the Phase-II to Phase-IV, which implies the importance of this methanogen in AD under high loading conditions. Recently, *Methanospirillum* has been found to play an important role in syntrophic propionate oxidation in phased anaerobic digestion (Maspolim et al., 2015c). As an increase in relative abundance was also observed for propionate oxidisers such as *Smithella*, the applied cascade system apparently provides proper conditions for attaining efficient syntrophic propionate conversion (de Bok et al. 2001).
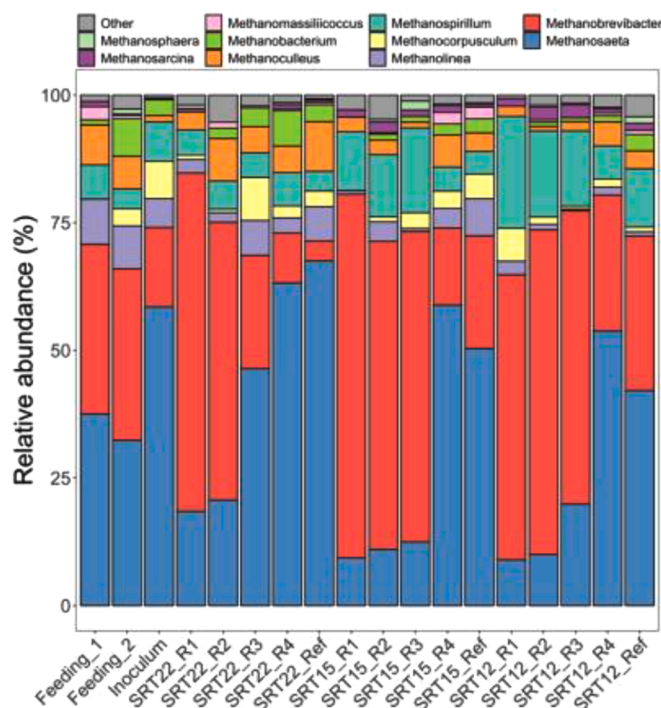

Fig. 9. Species taxonomy of methanogenic communities at the genus level. The species, whose sums of percentage in all the samples are less than 0.5%, are classified as "the others".

Conclusions

The conclusions drawn from the current work can be summarized as follows:

1. AD in the cascade system led to 8% more tCOD reduction than the single stage CSTR digester, both operated at a total SRT of 22 days. Stepwise reduction of the total SRT from 22 to 12 days did not affect the tCOD removal efficiency for the cascade system, but showed a 29% decrease in the tCOD removal in the reference-CSTR. Maintaining stability at high organic loading rates in a cascade system denotes an enhanced sludge treatment capacity of 30–35%, compared to a conventional sludge digester of the same volume.

2. Normalised specific hydrolysis rates, resembling the first-order hydrolysis rate constant, differed per reactor and increased with decreasing SRTs. The highest increase by a factor 2 was found in the individual reactors of the cascade system. Normalised hydrolysis increased by a factor 1.52 in the reference-CSTR.

3. Clear higher enzyme activities were found in the cascade system compared to the reference-CSTR, especially under short SRTs, which explains the overall accelerated specific hydrolysis rate in the cascade AD system. The overall hydrolytic enzyme activities increased with a factor up to 3 or even more, while this was a factor less than 2 for the specific hydrolysis rate, indicating that hydrolysis was limited by the solids-surface availability.

4. Several enzymes that target hydrolysis of SEPS-related organic compounds displayed reversed distribution and higher activity in the cascade system than in the reference-CSTR, indicating an additional degradation capacity of refractory compounds in the cascade system.

5. The increased relative abundance of key hydrolytic bacteria found in the first 3 reactors of the cascade system and the structural shift from hydrogenotrophic methanogens to acetoclastic methanogens alongside the cascade under low SRTs, demonstrated that cascading CSTRs possibly imposed selective pressures on the microbial

population, which contributed in achieving the enhanced enzymatic hydrolysis and sludge reduction.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117398.

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