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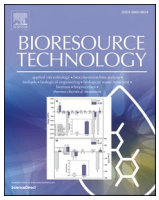

Case Study

Performance and microbial community composition of full-scale high-rate cascade sludge digestion system via pie-shaped reactor configuration

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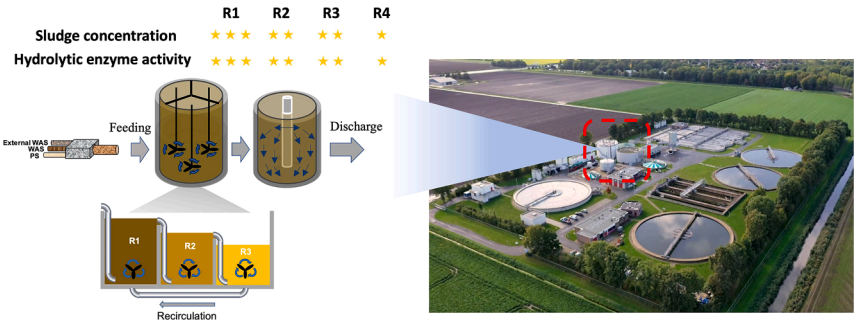
HIGHLIGHTS

- First full-scale, high-rate cascade sludge digestion system worldwide was built.
- Special pie-shaped reactor configurations were applied in the cascade system.
- Long-term high digestion efficiency was achieved with short sludge retention time.
- Enhanced hydrolytic enzyme activities were observed in the first compartment.
- First 3 compartments shared a similar community structure of hydrolytic consortia.

GRAPHICAL ABSTRACT



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ABSTRACT

A full-scale high-rate cascade anaerobic digestion (CAD) system was evaluated for its ability to enhance enzymatic sludge hydrolysis. The system included a newly built digester, innovatively divided into three pie-shaped compartments (500 m^3 each), followed by an existing, larger digester (1500 m^3). The system treated a mixture of waste activated sludge and primary sludge, achieving a stable total chemical oxygen demand reduction efficiency (56.1 ± 6.8 %), and enhanced sludge hydrolytic enzyme activities at a 14.5-day total solids retention time (SRT). High-throughput sequencing data revealed a consistent microbial community across reactors, dominated by consortia that govern hydrolysis and acidogenesis. Despite relatively short SRTs in the initial reactors of the CAD system, acetoclastic methanogens belonging to *Methanosaeta* became the most abundant archaea. This study proves that the CAD system achieves stable sludge reduction, accelerates enzymatic hydrolysis at full-scale, and paves the way for its industrialization in municipal waste sewage sludge treatment.

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1. Introduction

The conventional activated sludge (CAS) process is currently the most common biological wastewater treatment technology worldwide and extensively applied in wastewater treatment plants (WWTPs). CAS systems usually produce high amounts of waste sewage sludge, consisting of waste activated sludge (WAS) and primary sludge (PS) (Cao & Pawlowski, 2012). In the European Union alone, approximately 9 to 12 million tons of waste sewage sludge are produced annually (Eurostat, European Commission, 2022, link: https://ec.europa.eu/eurostat). Inappropriate use and poor treatment of waste sewage sludge may lead to odor nuisance and greenhouse gas emissions, leading to public and environmental health hazards. Therefore, proper sludge processing and adequate disposal are essential parts of wastewater treatment systems (Raheem et al., 2018).

Anaerobic digestion (AD) is one of the best-established processes applied to stabilize waste sewage sludge and achieve volume reduction and recovery of biochemical energy via biogas production. Unlike PS, which contains easily biodegradable cellulosic fibers, proteins, and fats, WAS contains more persistent extracellular polymeric substances and bacterial cells (Guo et al., 2020). Therefore, compared to PS digestion, WAS digestion often experiences lower hydrolysis rates and longer sludge retention times. This typically results in larger reactor volumes, which in turn leads to lower solids biodegradation rates and increased costs (Gonzalez et al., 2018). To overcome these drawbacks, several pre-treatment strategies to enhance hydrolysis in WAS have been developed, with thermal and acid/base treatments being the most reported (Zhen et al., 2017). While these strategies accelerate WAS hydrolysis rates, they also reveal the constraints related to excessive utilization of chemicals and/or energy inputs (Xu et al., 2020) and generation of refractory byproducts (Zhang et al., 2020). Compared to heating or chemically disrupting the sludge structure, enzymatic hydrolysis enhancement strategies have gained popularity in recent years. This is due to the mild process conditions and absence of the production of byproducts that may negatively affect downstream processing of the digestate (Jiang et al., 2021). Enzymatic hydrolysis enhancement can be realized by either additionally dosing active hydrolytic enzymes or by stimulating hydrolytic reactions /enzyme activities *in situ*. To date, most enzymatic WAS hydrolysis enhancement studies have been limited to lab-scale investigations for concept verification, with a pressing need for practical applications (Agabo-Garcia et al., 2019).

Enzymatic hydrolysis of WAS usually follows empirical first-order kinetics, where the conversion rate of solids depends on the substrate concentration and the hydrolysis rate coefficient. Therefore, by increasing the solids loading rate to a digester, higher conversion rates can be expected. Based on this principle, a very promising hydrolysis enhancement strategy has been developed recently, making use of a sequence of three small conventional completed stirred tank reactors (CSTRs), followed by large-sized CSTR, in a cascade configuration (Guo et al., 2021). With a total volume of the cascade AD (CAD) equal to a conventional AD, higher solids conversion rates and methane production rates were attained. Interestingly, observed hydrolysis rate coefficients in the first small-sized CSTRs was much higher compared to the reference CSTR, which was attributed to the increased hydrolytic enzyme activities that were measured in the small-sized CSTRs. The CAD system, which is currently marketed under the trade name Ephyra®, effectively accelerates hydrolytic enzyme activities during WAS digestion (Guo et al., 2021).

Full-scale sludge AD processes are commonly applied to co-digest WAS and PS, rather than to treat WAS alone (Ozgun, 2019). Previously, a lab-scale CAD system was studied fed with WAS alone, because the most significant effect of enzyme enhancement was expected for the typically slow-to-biodegrade WAS fraction (Guo et al., 2021). The effects of PS additions to WAS in a CAD system remains unexplored. However, results of Zhang et al. (2016) indicate that hydrolysis enhancement strategies, which are effective for WAS digestion, did not enhance PS digestion.

If two full-scale CSTR sludge digesters are available, a CAD process can be feasibly implemented by dividing one of the two digesters into three vertically divided smaller segments, while the second reactor is subsequently used as the final stage. Alternatively, if only one digester is available, a new compartmentalized digester can be constructed in front of the existing one. In the present work, the three so-called pie-shaped segments were connected in series to achieve the cascading effect and were subsequently followed by the second digester in series (Fig. 1). The assembled full-scale CAD system was fed with a PS/WAS mixture with a fluctuating composition, attributable to a fishing industry discharging wastewater to the WWTP and the high ratio between storm and dry weather flows. Degradation of the PS/WAS mixture was individually assessed for each reactor compartment by determining the organic solids reduction and volatile fatty acids production efficiencies, as well as the methane production rate. Additionally, the microbial community composition throughout the CAD process was analyzed using high-throughput sequencing.

2. Materials and methods

2.1. Description of the wastewater treatment plant and cascade anaerobic digestion system

The WWTP at Tollebeek receives approximately 20,000 and 100,000 m^3/day of municipal wastewater, during dry weather and stormwater flow, respectively. This WWTP consists of a two-stage activated sludge plant and a sludge digestion process with an annual treatment capacity of 4,600 t of dry solids. The location of the CAD system in the WWTP and its detailed process configuration are presented in Fig. 1a and b, respectively. The CAD system was composed of two digesters, both with a working volume of 1,500 m^3. One of the digesters was vertically divided into three mechanically mixed pie-shaped compartments of 500 m^3 each. A mixture of thickened PS (making up 40–45 % of the total flow, 15–48 g total solids (TS)/L), and WAS (making up 30–35 % of the total flow, 66–72 g TS/L) from the same WWTP, as well as a portion of external WAS (ES, making up 25–30 % of the total flow, 58–78 g TS/L) from the WWTPs Lelystad and Zeewolde, was fed to the first compartment, R1. Thereafter, the sludge mixture flowed to compartments R2 and R3 via a pipe overflow. From there, the mixture flowed to the second large digester, denominated as R4, where it was mixed via biogas recirculation. A controlled recycle flow from R3 to R1 with a low flow rate was used, based on the results of previous experiments for optimizing the CAD process (Guo et al., 2021). The level difference between the compartments was limited using equalization holes in the lower part of the vertical partitions. Finally, the digested sludge exiting R4 was dewatered in screw presses and transported to the final sludge disposal. The average SRT of each pie-shaped compartment was 2 days. During peak loadings, the SRT dropped to 1.7 days. The SRT of R4 was 8.5 days, resulting in a total system SRT between 13.6 and 14.5 days. The operating temperature of the entire CAD process was maintained at 37 ± 0.5 °C.

2.2. Sampling and analytic methods

The TS in both influent and effluent of the CAD system was measured according to standard protocols (APHA, 2005), and the total methane production of the entire process was measured via an industrial biogas flow meter equipped with a methane detector (Carbosys CH_4 CDE70, Endress + Hauser, Switzerland). PS, WAS, ES, and the effluent from each digester (R1-R4) were sampled bi-weekly, and six additional batch samples were collected between days 79 and 160. The six sampling batches (B1-B6) were used to determine the physicochemical characteristics of the sludge, i.e., the chemical oxygen demand (COD) including total COD (tCOD) and soluble COD (sCOD), ammonium (NH_4-N), and *ortho*-phosphate (PO_4-P) concentrations using standard test kits: LCK014

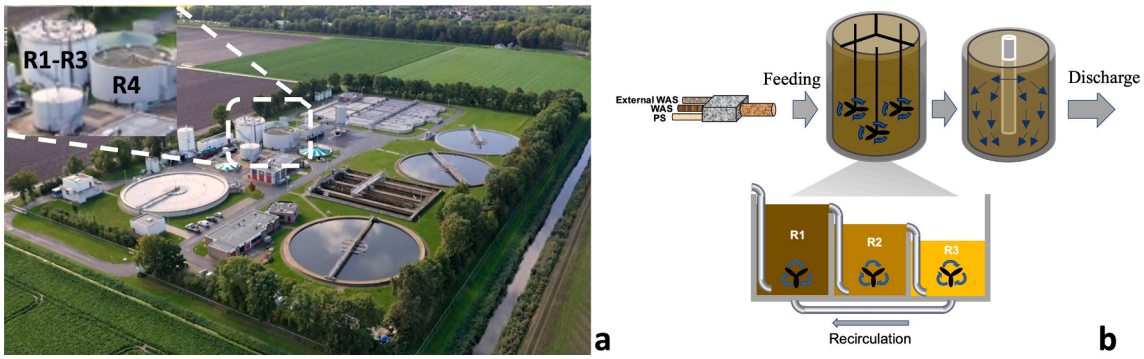

Fig. 1. The location of the full-scale CAD system in WWTP Tollebeek, the Netherlands (a), and schematic illustration of the CAD process (b).

for COD, LCK303 for NH_4-N, and LCK350 for PO_4-P (Hach Lange, Germany). Volatile fatty acids (VFAs) were measured using gas chromatography (GC) with a flame ionization detector (FID) (Agilent 7890A, USA). A 25 m × 320 μm × 0.5 μm column (Agilent 19091F-112, USA) was used, and helium was used as the carrier gas with a flow rate of 1.8 mL/min. The injection and oven temperature were 240 and 80 °C, respectively. The hydrolytic enzyme activities, including protease and cellulase, of sludge samples from B3 and B6 were analyzed to investigate the hydrolysis step in the CAD system. The activity of protease was determined using a Pierce fluorescent protease assay kit (Thermo Fisher, USA), and the activity of cellulase was determined using a fluorescent cellulase assay kit (MarkerGene, USA). All measurements were conducted using a 96-well microplate spectrophotometer (BioTek Synergy-HTX, USA). Triplicate sludge samples, including the influent and effluent from each digester of the CAD system, were collected for enzyme extraction on day 131. The hydrolytic enzymes were separated into free and sludge-attached fractions. Detailed information about these two enzyme fractions has been reported elsewhere (Guo et al., 2021).

During the experiment, the feed was sampled on days 100 and 145, and the digestates from R1, R2, R3, and R4, sampled on days 110, 141, and 155, were analyzed to evaluate the microbial community dynamics based on a procedure published previously (Guo et al., 2021). Briefly, a FastDNA® SPIN-Kit-for-Soil (MP Biomedicals, USA) was used to extract DNA according to the manufacturer's instructions, and the quality of the obtained DNA was verified using Qubit3.0 DNA detection (Qubit® dsDNA-HS-Assay-Kit, Life Technologies, USA). High-throughput sequencing and taxonomic assignment of the extracted DNA samples were performed at Novogene, UK. Raw sequence reads of the extracted DNA were deposited in the NCBI Sequence Read Archive database under accession number PRJNA1104005.

One-way ANOVA was applied using SPSS Statistics 25 (IBM, USA) to evaluate significant differences in chemical characteristics between the sludge samples. The significance level of probability (p-value) was set to 5 %.

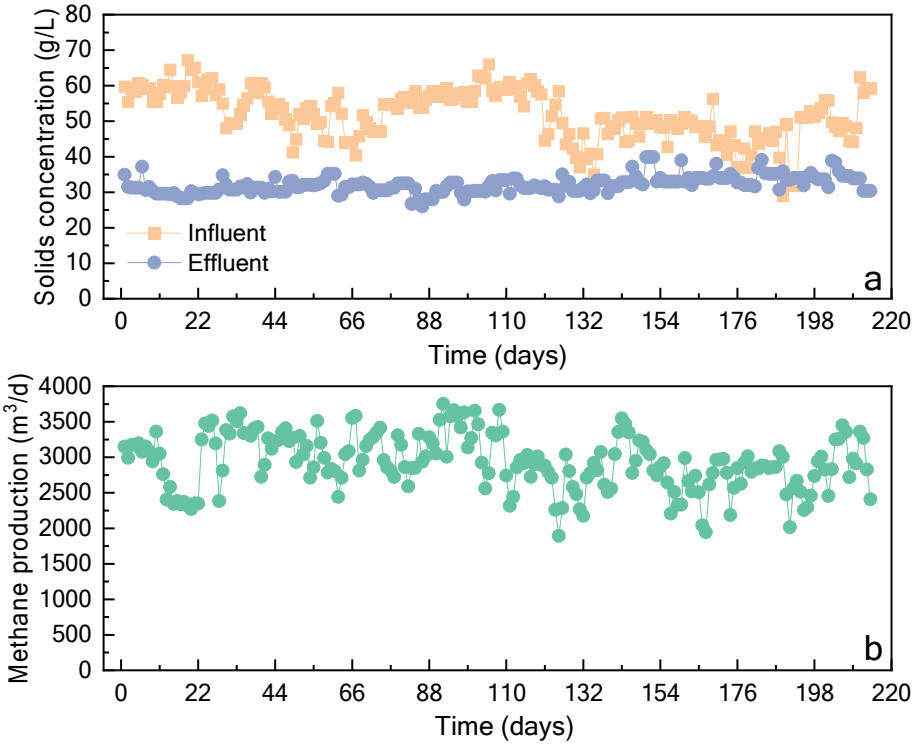

Fig. 2. Total influent and effluent solids concentration (a) and total methane production rate of the CAD system (b).

3. Results and discussion

3.1. Performance of the full-scale cascade anaerobic digestion process

The daily monitored total solids and methane production rate of the CAD system are shown in Fig. 2a and b, respectively. During the entire experimental period of 214 days, the total solids concentration of the influent sludge highly fluctuated between 28.4 and 68.2 g/L. Nevertheless, a stable total solids concentration in the effluent of approximately 30 g/L was determined during the entire study. The sludge reduction efficiency by the CAD system was 43.7 ± 10.9 % in TS, resulting in a total methane production of approximately 3,000 m^3/d. These results indicate that the CAD system could satisfactorily handle strong fluctuations in the solids concentration in the influent.

To better understand the different processes in the four compartments of the CAD system, the influent and effluent of each reactor compartment were sampled six times between days 79 and 160. The values of the measured TS, tCOD, sCOD, total VFAs, NH_4-N, and PO_4-P concentrations are presented in Fig. 3. The TS reduction determined based on these six samples showed a similar trend as that in the daily measurements, revealing that the highest TS reduction was obtained in R1 and R4, i.e, 25.5 ± 5.9 % and 16.5 ± 2.1 %, respectively. The results revealed a tCOD conversion of 56.1 ± 6.8 %, a declining percentage throughout the cascade process from 70.2 ± 11.6 g/L in R1 to 30.1 ± 0.9 g/L in R4 (Fig. 3a and b). In line with the TS reduction, the largest decrease in tCOD concentrations occurred in R1, in which more than 70 % of the tCOD conversion took place. R2 and R3 only contributed to a tCOD reduction of approximately 4 % each. This observation is in line with the ammonia profile that followed a reciprocal trend (Fig. 3a and e). The results indicate that R1 had the highest conversion rate for protein compounds. The phosphate concentration also increased sharply in R1 but stabilized in the subsequent reactors and even slightly decreased in R4 (Fig. 3f). A possible explanation for the observed dynamics is the release of phosphate by phosphate-accumulating organisms in the first compartment, which coincided with high VFA concentrations in the influent and sludge in R1 (Fig. 3d), whilst phosphate precipitation likely occurred in R4.

The results obtained in this full-scale measuring campaign were compared with those obtained in the laboratory scale CAD system (Guo et al., 2021). In the latter work, the CAD system was operated at a similar total SRT of 12 to 15 days, while only 8.1 % of the tCOD was converted in R1, and 9.0, 7.7, and 14.3 % of tCOD was converted in R2, R3, and the larger digester R4, respectively. A major difference between the two systems is that the full-scale installation was fed with a mixture of PS and WAS, while the laboratory-scale system was fed solely with WAS from a nearby WWTP. Typically, the conversion rate, especially hydrolysis rate, of PS is approximately 1.4 times higher than that of WAS during AD, due to the high content of rapidly biodegradable mass, such as cellulosic fibers and lipids (Guo et al., 2020). This difference between PS and WAS could have been one of the reasons for the observed enhanced sludge hydrolysis in R1 of the full-scale CAD. Notably, the influent of the full-scale system contained a high fraction of readily biodegradable COD, i.e., 4.9 g/L, of which on average 2.1 g/L were VFAs (Fig. 3c and d). This readily biodegradable COD is likely immediately converted in the first compartment (R1), contributing to the observed high tCOD conversion. Unfortunately, the methane production of the individual compartments could not be determined in the full-scale installation; the produced biogas of all compartments was collected, and the total volume was

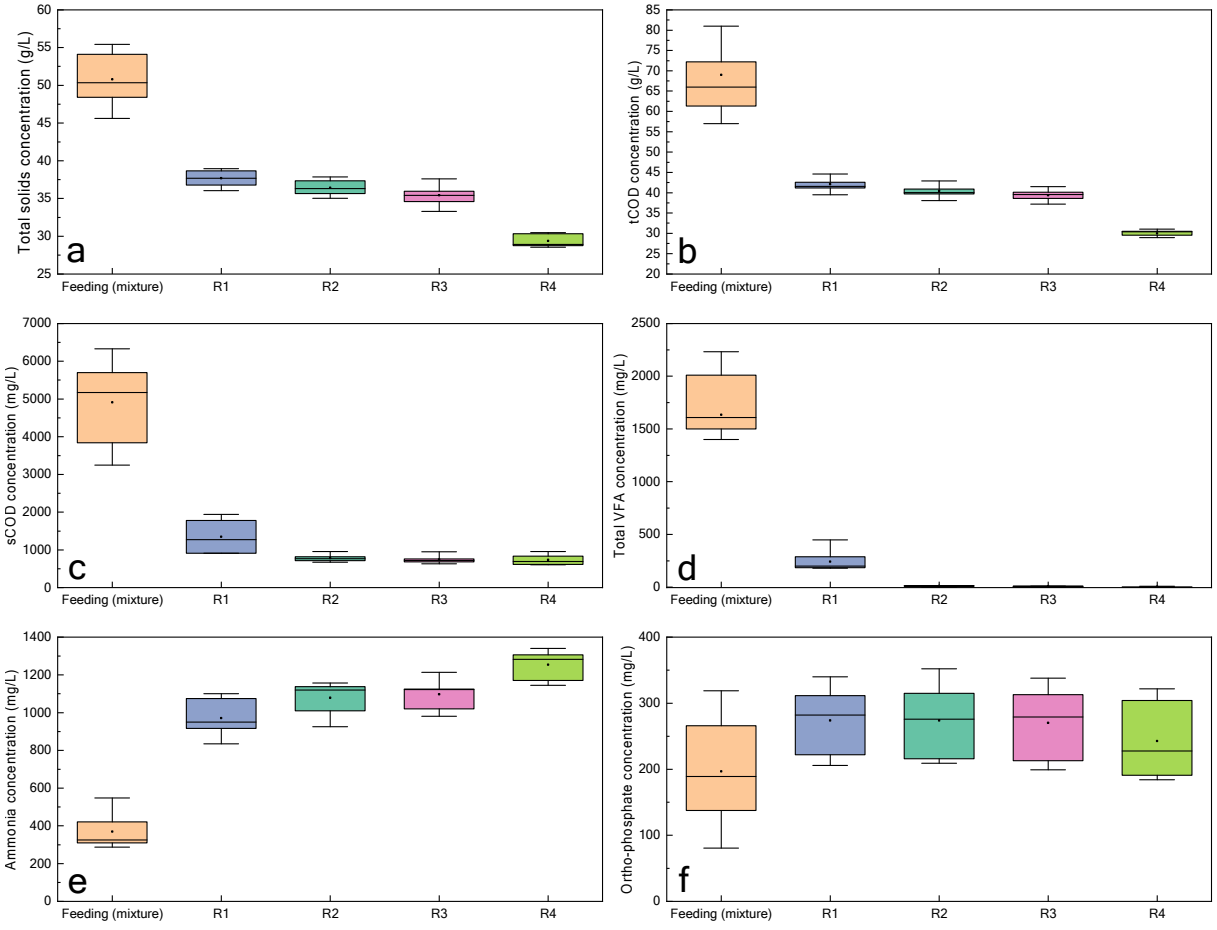

Fig. 3. Chemical characteristics of each reactor, total solids concentration (a), tCOD concentration (b), sCOD concentration (c), total VFA concentration (d), ammonia concentration (e), and *ortho*-phosphate (f). Error bars refer to the standard deviation (n = 18, with triplicates for each sample).

measured using a single device. Therefore, it was not possible to determine the specific hydrolysis rate per compartment in the full-scale CAD system. Cellulosic fibers and proteins are widely recognized as the predominant organic components in both municipal WAS and PS (Gonzalez et al., 2018). For example, the average cellulosic fiber and protein contents of WAS and PS sampled from a typical Dutch municipal WWTP are, respectively, 90 and 389 mg/g VS sludge (for WAS), and 180 and 248 mg/g VS sludge (for PS) (Guo et al., 2020). Therefore, instead of measuring the specific hydrolysis rate, cellulase and protease activities were selected as representative enzyme activities to characterize the hydrolysis rate of sludge in each reactor of the CAD system (Fig. 4). R1 presented the highest activity of both enzymes, with values of 10.0 ± 0.8 U/mL for protease and 0.1 ± 0.0 U/mL for cellulase, followed by R2 and R3, while R4 had the lowest enzyme activity. The used substrates showed much lower protease and cellulase activities than the sludge samples from each reactor (Fig. 4), demonstrating high enzyme induction in the studied CAD process. Compared to the lab-scale CAD system (Guo et al., 2021), the protease and cellulase enzyme activities were comparable in the first compartment R1. The trend along the cascade somewhat differed, with higher enzyme activities in R2 and R3 in the lab-scale reactor than in the full-scale CAD system. The tCOD loading rate of R1 in the full-scale CAD was similar to that of R1 in the laboratory-scale CAD at a 15-day SRT (a 1.5-day SRT specifically in R1), indicating similar hydrolytic enzyme activities in R1 of both full-scale and laboratory-scale CAD processes. These results clearly show accelerated enzymatic hydrolysis of sludge using the cascade digestion configuration at full scale.

3.2. Microbial community composition and dynamics

In the laboratory-scale CAD system, a clear differentiation of the microbial population between the different compartments was observed (Guo et al., 2021). To determine whether this was the case in the full-scale system, the microbial community structure of the different compartments of the CAD system was analyzed and compared with that in the influent sludge. Three sampling batches (day 110, 141, and 155) were analyzed using high-throughput gene sequencing. Approximately 2.2 million high-quality sequences were generated from all sludge samples and assigned to OTUs. Duplicates of each sludge sample were clustered in a PCA plot (Fig. 5). The results show that R1, R2, and R3 samples of each batch shared a similar microbial community structure. A distinct difference in the microbial community structure between R4 and R1 to R3 was expected owing to the relatively long SRT applied in R4 (Baldi et al., 2019; Wang et al., 2018). However, there was no remarkable difference between R4 and the other compartments.

A detailed examination of the microbial composition of all sludge samples at the genus level revealed that the three types of feed sludge contained different dominant groups (Fig. 6a). The WAS possessed a high relative abundance of nitrifiers and phosphate-accumulating organisms, such as *Nitrospira* and *Candidatus_Accumulibacter* (Rubio-Rincon et al., 2017; Zhang et al., 2015), as can be expected for activated sludge from a modified University of Cape Town system. PS was dominated by facultative *Ottowia*, *Trichococcus,* and Arcobacter as well as strictly anaerobic *Macellibacteroides* (Mei et al., 2016). The ES that is initially (anaerobically) stored until transport to WWTP Tollebeek, showed species commonly associated with denitrification, *Kouleothrix*

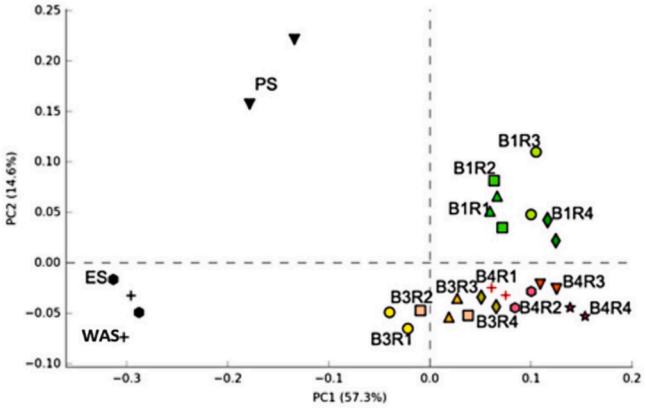

Fig. 5. Microbial principal component analysis (PCA). All samples were measured in duplicate.

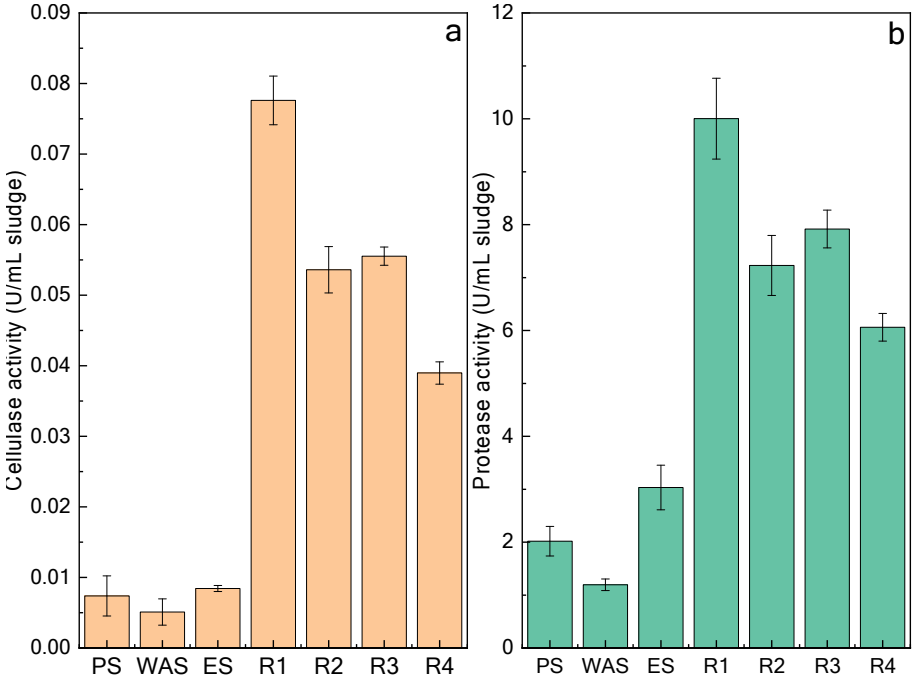

Fig. 4. Enzyme activity in U per mL of sludge from the feeding and each reactor compartment, sludge-attached cellulase (a), and sludge-attached protease (b). Error bars refer to the standard deviation (n = 6, with triplicates for each sample).

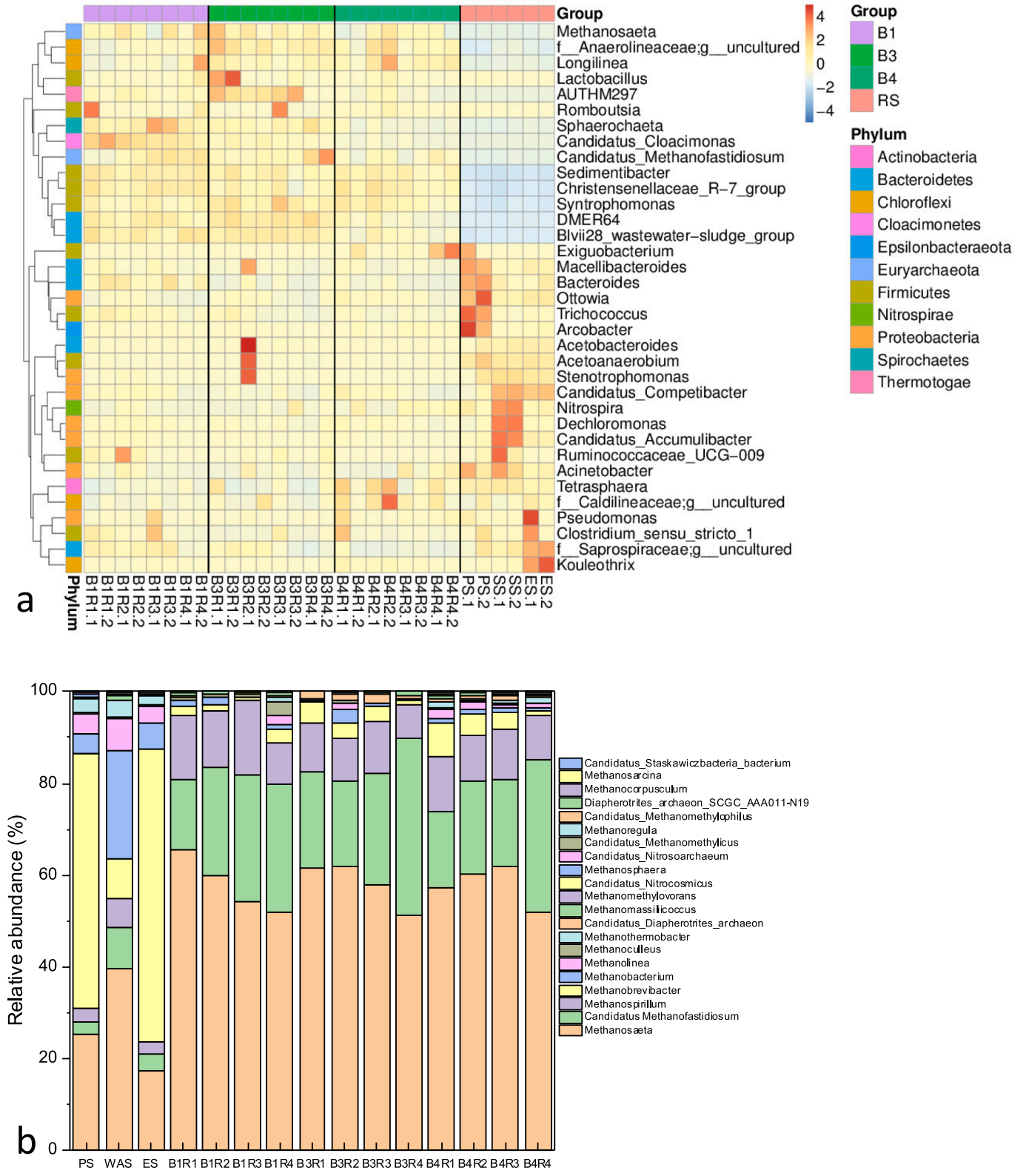

Fig. 6. Normalized relative abundance of taxa related to the dominated microbes of interest (top 35 species that presented in both feeding and reactors) at the genus level (a), and species taxonomy of methanogenic communities at the genus level (b).

and *Pseudomonas* (De Sotto & Bae, 2020), and *Clostridium_sensu_stricto_1*, which is known as anaerobic sugar and protein fermenter (Luo et al., 2020). The mixing of three sludge types and the solids hold-up in the first step of the cascade (R1) significantly reduced the relative abundances of the predominant genera in the individual sludge types (p-value < 5 %). The most abundant bacteria on the genus level in R1, ranked by relative abundance, were *Candidatus_Cloacimonas*, *DMER64*, *Sedimentibacter*, *Christensenellaceae_R-7_group,* and *Syntrophomonas*, which have been reported to regulate hydrolysis and acidogenesis of sludge in sludge digesters (Lee et al., 2018; Shakeri Yekta et al., 2021).

Despite a slightly higher relative abundance of these genera in R1 and R2 than in R3 and R4, no clear changes in the bacterial fingerprints of the digestate from R1 to R4 were observed, irrespective of samples from different batches. This finding supports the results of the PCA and indicates that the microbial fingerprint of CAD process did not reflect the key microorganisms in PS, although the addition of PS in WAS could have enhanced the tCOD reduction in CAD process (Fig. 3b).

Two genera affiliated to the phylum Euryarchaeota, i.e., *Methanosaeta* and *Candidatus Methanofastidiosum*, were substantially enriched in all digesters and sampling times (Fig. 6a). Fig. 6b indicates that *Methanosaeta* and *Candidatus Methanofastidiosum* accounted for approximately 60 and 15 % of the abundance of archaea in R1 to R3, and 50 and 20 % of the abundance of archaea in R4, respectively. *Methanosaeta* are typical acetotrophic methanogens that strictly metabolize acetate as their sole source of energy (De Vrieze et al., 2012). In multi-stage digesters and CAD systems, hydrogenotrophic methanogens generally proliferate in the first high-loaded reactor(s) (Shimada et al., 2011; Guo et al., 2021), while *Methanosaeta* proliferates under low loadings of non-hydrolyzed and non-acidified COD. Notably, the maximum growth rate (u_{max}) of *Methanosaeta* is 3 to 9 times lower than that of hydrogenotrophic methanogens (Pavlostathis, 2011). Considering the high acetate concentrations in R1, *Methanosaeta* likely grew at its maximum rate, even though the SRT in each reactor is too low to maintain this slow-growing archaea. It is hypothesized that the high relative abundance of *Methanosaeta* might be related to the digestate recirculation strategy in the CAD system. In this study, digestate recirculation from R3 to R1 was applied, with the main aim to enhance the buffer capacity of R1 that faced high VFA concentrations (Fig. 3d) (Guo et al., 2021). Simultaneously, the applied recirculation also recycled biomass and key bioactive micro-nutrients, stimulating the sludge degradation process in R1-3. Biomass recirculation resulted in an extension of the actual SRT in these reactors, favoring the net-growth of *Methanosaeta* in the sludge mass. The obtained results were possibly also affected by the design of the full-scale CAD system; the equalization holes in the partitioning walls could have led to additional biomass exchange between R1, R2, and R3. Furthermore, the pie-shaped reactors R1-3 were possibly characterized by inefficient mixing and poorly mixed zones, affecting the actual SRT in the reactors (Angelidaki et al., 2005). An increased degree of mixing between all reactors was also evidenced by the similar microbial community structure in all reactors (Fig. 5), implying that the exact SRT of R1-3 might have been much longer than expected. Notwithstanding the relatively high abundance of *Methanosaeta* in the first compartments of the full-scale CAD system, sludge degradation remained at a high level. This situation also prevailed when a high solid loading rate or a short SRT was applied. Future research should focus on the mixing patterns in different compartments, while having provisions for measuring methane production in each separate compartment, to further characterize the different stages of the full-scale CAD.

Members of the genus *Candidatus Methanofastidiosum* are recently described as the sixth class of euryarchaeotal methanogens and their exact function in sludge AD process is not yet clear. *Candidatus Methanofastidiosum* is characterized as a versatile methanogen that has the capacity to produce methane from H_2/CO_2, acetate and methanol (Nobu et al., 2016). Meanwhile, this species can only inhabit ecosystems with other active methanogens (Nobu et al., 2016). The observed presence and dynamics in relative abundance of this genus in the studied CAD process were possibly related to its functionality as hydrogen scavenger for efficient fermentation (Sitthi et al., 2022), next to acetotrophic methanogenesis for which that *Methanosaeta* was mainly responsible (Tonanzi et al., 2021).

4. Conclusion

Despite fluctuating sludge loads, the CAD process using a pie-shaped reactor configuration and a minimal digestate recirculation ratio consistently achieved a tCOD reduction of approximately 56 % and showed increased protease and cellulase activity in the initial compartments. Microbial analyses indicated a prevalence of fermentative bacteria in the first three compartments, along with the dominance of *Methanosaeta* species as the primary archaea. This dominance can be attributed to the possible increased SRT in R1-3, resulting from digestate recirculation, equalization holes, and the mixing characteristics of the pie-shaped reactor configuration. .

CRediT authorship contribution statement

Hongxiao Guo: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Maaike McIntyre:** Writing – review & editing, Project administration, Conceptualization. **André Visser:** Writing – review & editing, Project administration, Funding acquisition. **Hans Kuipers:** Writing – review & editing. **Jules B. van Lier:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Merle de Kreuk:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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