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Achieving expanded sludge treatment capacity with additional benefits for an anaerobic digester using free ammonia pretreatment

Huan Liu [a], Xuan Li [a], Zehao Zhang [a], Long D. Nghiem [a], Li Gao [b], Damien J. Batstone [c], Qilin Wang [a,*]

[a] Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney, Ultimo, NSW 2007, Australia
[b] South East Water, 101 Wells Street, Frankston, VIC 3199, Australia
[c] Australian Centre for Water and Environmental Biotechnology (ACWEB, Formerly AWMC), The University of Queensland, St Lucia, QLD 4072, Australia

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ABSTRACT

Population growth rapidly increased waste activated sludge (WAS) production in wastewater treatment plants (WWTPs), making the expansion of sludge treatment capacity urgent. Free ammonia (FA) pretreatment is experimentally applied to expand the treatment capacity of an anaerobic digester through reducing sludge retention time (SRT) for the first time. Two semi-continuous flow mesophilic (37 °C) anaerobic digestion systems, control system with a uniform SRT of 12 d and the experimental systems with progressively reduced SRTs (from 12 d to 10 d and then 8 d), were operated for>7 months. The volatile solids (VS) destruction in the experimental system at a SRT of 8 d was comparable to the control system (30.0 ± 1.4 % *vs* 30.5 ± 1.7 %) but increased by 16.2 % (35.1 ± 1.5 % *vs* 30.2 ± 1.4 %) under an SRT of 10 d, which was supported by methane production and total chemical oxygen demand (COD) removal. The biomass-specific hydrolysis rate was significantly increased by up to 80 % (from 0.05 ± 0.01 g COD/g VS/d to 0.09 ± 0.01 g COD/g VS/d), which may contribute to the expanded capacity. The volatile fatty acids (VFAs)/alkalinity of systems maintained a reasonable range (0.01 – 0.06), suggesting the stability of digesters. FA pretreatment played a dominant role in the changes in the bacterial microbial community (52.80 % in PC1) and archaeal community (94.25 % in PC1). FA pretreatment improved the removal of pathogen by 1.3–2.0 log and antibiotic resistance genes by 34–86 %. This study first demonstrated that FA pretreatment expands the treatment capacity of an anaerobic digester by up to 50 % with economic and environmental benefits, promoting FA pretreatment to be a wider and pragmatic implementation for WWTPs.

1. Introduction

Waste activated sludge (WAS) is an undesirable by-product of the biological wastewater treatment process. Its treatment and disposal cost up to 60 % of the whole operation expenditure in wastewater treatment plants (WWTPs) [1,2]. Anaerobic digestion (AD) is widely implemented for WAS treatment in WWTPs, which destroys and stabilizes solids, and generates biogas for energy recovery [3,4]. Due to the low, and slow biodegradability of WAS, a long sludge retention time (SRT) of 12–30 d is commonly applied for AD, which limits the daily sludge treatment capacity of the digester [5–7]. Furthermore, the rapid increase of WAS production due to population growth substantially challenges the AD treatment capacity and adds operation and upgrading costs to WWTPs, especially for the WWTPs with constrained capacity. Therefore, techniques to increase the sludge treatment capacity of AD are needed to accommodate population growth and minimize the operational costs of WWTPs.

In AD treatment of WAS, hydrolysis is believed the rate-limiting step [8,9]. To improve the hydrolysis rate of WAS, a variety of pretreatment technologies have been applied to AD, including chemical, physical and biological technologies [10–12]. However, these pretreatment technologies all require additional chemicals or energy as input. The merits of free ammonia (FA, NH_3) pretreatment have been widely investigated and reported by our group, which has shown promising results in enhancing AD treatment efficiency [13,14]. Through biochemical methane potential (BMP) tests, FA pretreatment of WAS for 24 h increased the hydrolysis rate by 140 % and improved biochemical methane potential by 22 % at FA concentrations of 420 – 680 mg NH_3-

* Corresponding author.
E-mail address: Qilin.Wang@uts.edu.au (Q. Wang).

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N/L [13]. The improved hydrolysis for sludge was mainly due to the enhanced degradation of extracellular polymeric substances (EPS) and/or cells via FA pretreatment [15]. Similar results were achieved in semi-continuous flow systems, where FA pretreatment at 560 mg NH_3-N/L for 24 h improved the WAS volatile solid (VS) destruction by 26.4 % and methane production by 28.6 % [15]. The improved VS destruction and hydrolysis rate identify the potential of FA pretreatment to increase the capacity of the anaerobic digester. Thus, we hypothesize that FA pretreatment may maintain AD performance with a reduced SRT. Therefore, FA pretreatment potentially provides an effective solution for accommodating the increase in WAS production and saving the upgrading costs of WWTPs.

However, the FA pretreatment has never been experimentally evaluated in expanding the treatment capacity of an anaerobic digester. The AD process is a biological process, relying on the microorganism activities. Expanding treatment capacity of an AD system by FA pretreatment will require a reduced SRT, which may cause the instability of an AD system. Also, the essential performance of an AD system applied FA pretreatment with a reduced SRT is still unknown, such as the VS destruction and methane production. Additionally, AD also plays a critical role in stabilizing sludge and minimizing environmental risks, as over 70 % of sludge is used as biosolids in agriculture [8]. Although FA pretreatment showed effective performance in the pathogens and antibiotic resistance genes (ARGs) removal [15,16], the performance of FA pretreatment in the eliminations of pathogens and ARGs under the shortened SRT, as proposed in this study, is largely unknown. Therefore, these investigations are essential and substantial for the real application of FA pretreatment in WWTPs, which will provide an indispensable and comprehensive evaluation of FA pretreatment strategy for expanding AD capacity (i.e. at shortened SRT).

In this study, we investigated whether FA pretreatment can expand the treatment capacity of an anaerobic digester and comprehensively assessed its performance and benefits along with capacity expanding for the first time. Two sets of semi-continuous flow AD systems were operated for>7 months. Following system convergence, FA pretreatment was implemented for the experimental reactor with the SRT shortened from 12 d to 10 d and 8 d. The control system was operated with an SRT of 12 d throughout the study period. The system performance and stability, including VS destruction, total chemical oxygen demand (TCOD) removal, methane production, digester stability, biomass specific hydrolysis rates, microbial community, as well as ARGs, and pathogen removal, were evaluated. The study first provided a comprehensive feasibility assessment of upgrading the capacity of the anaerobic digester by using FA pretreatment. This study also boosts the implementation of FA pretreatment to a more integrated extent and provides a potential resolution for WWTPs to accommodate an increasing amount of sludge.

2. Materials and methods

2.1. Sludge sources

Both the inoculum (anaerobically digested sludge) and thickened WAS were collected from a local wastewater treatment plant (WWTP) in Australia that receives local wastewater at a flow of approximately 11000 m^3/day on average. This WWTP is a biological nutrient removal plant with an SRT of 15 days. The thickened WAS was obtained from the thickener following the secondary clarifier. The inoculum was obtained from a mesophilic (37 °C) AD system fed with WAS and primary sludge and operated at an SRT of 15 days. The key characters of inoculum and WAS are shown in Table 1. The thickened WAS was obtained fortnight and placed in a refrigerator (4 ± 1 °C) and diluted before use (as described in section 2.2). It should be noted that the SCOD of the thickened WAS in our study (i.e. 2.1 ± 0.5 g COD/L) is a bit higher than that of the normal thickened WAS, which may be due to the pre-fermentation during the WAS storage because the thickened WAS was collected fortnightly. This should be noted by readers while evaluating the WAS hydrolysis.

Table 1
Characteristics of the waste activated sludge (WAS) and inoculum.

Characteristic	Inoculum	Waste activated sludge
Total solids (TS, g/L)	17.3 ± 0.1	23.2 ± 2.1
Volatile solids (VS g/L)	13.0 ± 0.1	18.3 ± 1.9
Total chemical oxygen demand (TCOD, g/L)	20.0 ± 0.5	28.7 ± 0.7
Soluble chemical oxygen demand (SCOD, g/L)	1.0 ± 0.2	2.1 ± 0.5
Total Kjeldahl nitrogen (TKN, g N/L)	2.0 ± 0.1	2.5 ± 0.3
pH	7.5 ± 0.1	6.5 ± 0.1
Alkalinity (g $CaCO_3$/L)	2.6 ± 0.2	0.1 ± 0.1

2.2. Experiment design and operation

Two mesophilic AD systems (37 °C) (i.e., control and experimental systems) were established using two sealed reactors with 1 L working volume (1.4 L volume in total) and water-jacketed glass each, as shown in our previous study [15]. Briefly, the sealed reactors were maintained at around 37 °C through the heated and cycled water with simultaneous sludge feeding and discharging through peristaltic pumps. Primarily, the reactors were seeded with inoculum (1 L) and then flushed with nitrogen for 30 min to create an anaerobic atmosphere. WAS and pretreated WAS were fed into the control and experimental reactors daily. The reactors applied magnetic stirring (410–430 rpm) with daily monitoring of pH. The daily production of biogas was monitored by bucket gas meters linked to the headspace of the AD system. The discharged biogas from the bucket gas meters was stored via aluminum-foil gas bags for component and content analysis. The two digestion systems were operated in three stages: Baseline, Stage 1, and Stage 2. Pretreatment was implemented in the experimental reactor as previously described [15]. Briefly, total ammonia nitrogen (TAN, 960 mg N/L) and WAS were added to the closed pretreatment reactor (250 ml Erlenmeyer flask) every day, and the pH was raised to 9.5 ± 0.1 to achieve the target FA level of 560 mgNH_3/L [15]. The WAS was pretreated in the closed pretreatment reactor with magnetic stirring (430 rpm) for 24 h. It should be noted that this study aimed to demonstrate the feasibility of the FA pretreatment in expanding the sludge treatment capacity. Therefore, the optimization of FA pretreatment was not conducted. This was done at room temperature (22 ± 2 °C), and FA concentrations determined using the temperature corrected $K^+_{a,NH4}$ as previously described [15].

2.2.1. Baseline stage (Day 1–75)

Both systems run at a sludge retention time (SRT) of 12 d with a sludge fed rate of 83 ± 1 ml/d. Sludge was synchronously discharged at the same flow rate. Pretreatment was not implemented in the two systems. The two systems were run in parallel to reach stability, which was indicated by stable VS destruction and methane production in the two systems. The Baseline stage lasted for 6 SRTs.

2.2.2. ***Stage 1*** *(Day 76–143)*

The control system was run as same as in the Baseline stage without any pretreatment under an SRT of 12 d. In the experimental system, WAS was pretreated using FA at a concentration of 560 mg NH_3-N/L for 24 h before feeding. The treated WAS was fed into the experimental system at a rate of 100 ± 2 ml/d, achieving an SRT of 10 d. Stage 1 lasted for>6 SRTs for the experiment system.

2.2.3. ***Stage 2*** *(Day 144–223)*

The control system run as same as that in the Baseline stage, with an SRT of 12 d without pretreatment. In the experimental system, WAS was pretreated by FA and fed at a rate of 125 ± 2 ml/d into the digester, achieving an SRT of 8 d. Stage 2 lasted for>6 SRTs for the experiment

system.

An alternative experimental design is to add another control system for stages 1 and 2 using the shortened SRT (i.e. 10 d and 8 d), without FA pretreatment. However, this study aims to provide an alternative solution to expand the digester capacity when WWTPs are facing a growing amount of WAS. A shortened SRTs of 10 d and 8 d would be unlikely to be implemented without pretreatment and the current experimental design effectively assesses alternative strategy *vs* the minimum baseline of 12 d untreated. The basic effect analysis has been previously conducted in Liu et al. [15], with the main objective here being the impact of reduced SRT in the experimental one.

The VS and total chemical oxygen demand (TCOD) of the feed and anaerobically digested sludge (ADS) from the systems were obtained twice a week. The SCOD was measured once a week, and ammonium concentrations were measured twice per week in the two systems. Alkalinity and the volatile fatty acids (VFAs) concentrations were tested every week to ensure that both systems maintained steady AD. Biogas production was obtained every day by bucket gas meters linked to the reactors. The biogas components were analyzed when the two systems achieved a steady performance in each stage. The biomass specific hydrolysis rate was obtained every week during the steady period of stages 1 and 2. For microbial community analysis, replicate inoculum samples were collected on day 0 before feeding into the digesters (i.e. Inoculum_a and Inoculum_b). Replicate digested sludge samples were collected on days 105 (stage 1) and 190 (stage 2) from control and experimental reactors, namely FA_S1_a & b, FA_S2_a & b and Con_S1_a & b, Con_S2_a & b, respectively. The pathogen indicators, including Fecal Coliforms and *E. Coli,* in the feed and ADS through FA pretreatment or not were tested in replicates in the steady periods of stage 1 and stage 2. For ARGs analysis, replicate samples were collected on day 105 (steady stage 1) and 190 (steady stage 2) in WAS, discharged sludge from the experimental and the control reactors.

2.3. Analytical methods

TS and VS of feeding and digested sludge, soluble chemical oxygen demand (SCOD), ammonium nitrogen (NH_4^+-N), TCOD, and total Kjeldahl nitrogen (TKN) were obtained according to the standard method [17]. To measure soluble factors, the sludge samples were first centrifuged at 10,000 r/min for 10 min, afterwards the supernatant was filtered using syringe filters (0.45 μm) before measurement. The concentrations of VFAs and biogas composition were obtained by a gas chromatography (7820A, Agilent, USA). Biogas production was obtained by a bucket gas meter (3.2 ml/bucket, Ritter, Germany) that were linked to the reactors. VS destruction and TCOD removal of the digesters were calculated according to the equation R destruction (%) = (R_{in}-R_{out})/R_{in} × 100 %, where R_{in} and R_{out} represent the VS or TCOD concentration in the fed and discharged sludge.

'Refractory' SCOD is measured to estimate how the FA pretreatment at shortened SRTs can potentially affect the effluent COD concentration after the digestion liquor is returned to the wastewater treatment line of the WWTPs [18,19]. 'Refractory' SCOD was determined using modified Zahn-Wellens tests under aerobic conditions. Briefly, anaerobically digested sludge from the systems was centrifuged at 6000 rpm for 20 min, and then the supernatants were filtered with polyester filters (0.45 μm, Glass Vials Australia). The filtrate was then used as a substrate for the modified Zahn-Wellens test [20]. Aerobic inoculum (WAS) was washed 3 times with tap water before being added to the substrate. Then, the substrate with aerobic inoculum was aerated for 3 h to simulate the actual hydraulic retention time for COD removal in the wastewater treatment process. To eliminate any influence from the inoculum, a blank group was also operated, replacing the substrate with tap water. The remained SCOD from the substance subtracting the SCOD from the blank group after three hours of aeration was regarded as 'refractory' SCOD.

The biomass specific hydrolysis rate of a system was calculated according to Guo et al. [21].

$$\text{Biomass specific hydrolysis rate (g COD/g VS/day)} = (\text{mass\_sCOD}_{\text{out}}\text{n} + \text{nmass\_SCOD}_{\text{CH4}} - \text{mass\_sCOD}_{\text{in}})/\text{mass\_VS}_{\text{reactor}} \quad (1)$$

where mass_$sCOD_{out}$ and mass_$sCOD_{in}$ indicate the amount of sCOD per day in the discharging sludge and feeding sludge of the reactor (g/day), respectively; mass_$SCOD_{CH4}$ is the amount of produced CH_4 calculated as COD per day from the reactor (g/day); and mass_$VS_{reactor}$ represents the amount of biomass in the reactor (g VS). It should be noted that the FA pretreatment reactor and the anaerobic digester were treated as a whole system to compare with the control system in the calculation.

The DNA extraction and sequencing details are shown in the Supporting Information. The microbial genomic DNA of the sludge samples was extracted using FastDNA Spin Kit for Soil (MP Biomedicals, USA), according to manufacturer's instructions. The V3-V4 regions in 16S rRNA genes were amplified using 338F-806R primer pair for bacteria, while V4-V5 regions of 16S rRNA genes were amplified using 524F10extF-Arch958RmodR primer pair for archaea. The amplified DNA was analyzed using Illumina Miseq sequencing. Raw sequencing reads were analyzed using QIIME 2 pipelines (version 2020.2) to pair forward and reverse sequences. The operational taxonomic units (OTUs) were clustered using DADA2/Dcblur at 97 % similarity truncated values with chimeric sequences removed. The taxonomic assignment was performed against the SILVA 138 database with a minimal 70 % confidence estimate.

Pathogen removal was tested as the methods described in our previous study [15]. Briefly, the samples were firstly diluted by 100–100,000 times and then added Colilert®-18 reagents following the instructions. Then, the mixture was sealed in Quanti-Tray®/2000 and placed at 44.5 ± 1 °C for 18 h. Finally, Most Probable Number (MPN) of *Fecal Coliform* and *E. coli* in the sludge was obtained according to the quantity of positive grids and the MPN Table provided by IDEXX Quanti-Tray®/2000.

For ARGs, replicate samples collected at each stage was mixed and used for extracting DNA using FastDNA Spin Kit for Soil (MP Biomedicals, USA) [16]. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to obtain the abundance of the targeted genes. qPCR information was detailed in Test S1. For target genes, one aminoglycoside and fluoroquinolone resistance gene (aac(6′)-Ib-cr), one sulphonamide resistance gene (sul1), one beta-lactamase resistance gene (blaTEM), and two tetracycline resistance genes (tetA and tetX) were selected. These ARGs were targeted due to their common occurrence in sludge samples and also represented various antibiotic classes that are extensively applied in human activity. The primers are listed in the Table S2. The absolute abundances of the aimed genes were shown by gram of TS (i.e., gene copies/g-TS).

2.4. Statistic analysis

The microbial community information was visualized by heatmaps through R (version 4.2). Principal coordinates analysis (PCoA) was conducted by *R*-3.3.31 (vegan). Difference between the experimental and control systems was tested by *t*-test, with a significance threshold of 0.05.

3. Results

3.1. VS destruction

The results of VS destruction, TCOD removal, and TAN (NH_4^++NH_3-N) generation in three stages, Baseline, stage 1 and stage 2, are shown in Fig. 1.

In the baseline period (Day 1–75), the two systems reached convergence around Day 38 with comparable ($p > 0.05$) VS destruction (31.0

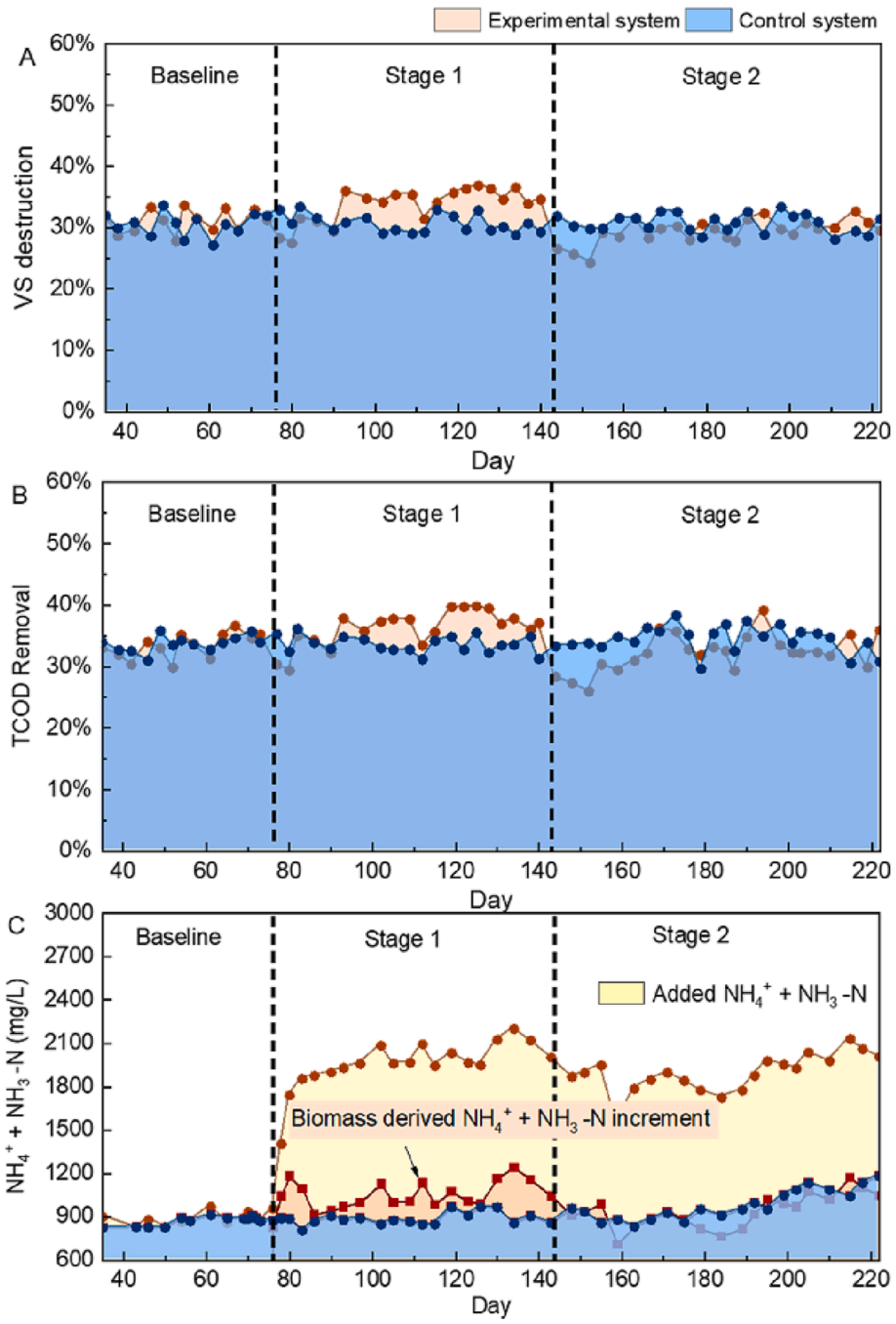

Fig. 1. Performance of the experimental and control systems. (A) VS destruction; (B) TCOD removal; (C) TAN concentration (NH_4^++NH_3-N); Biomass-derived NH_4^++NH_3-N increment was the TAN due to VS destruction in the experimental system. Added NH_4^++NH_3-N was the added TAN by FA pretreatment.

± 2.0 % in the experimental and 30.4 ± 1.9 % in the control system, Fig. 1A and Table 2), and TCOD removal (33.4 ± 2.2 % in the experimental and 33.7 ± 1.4 % in the control system, Fig. 1B and Table 2) from day 39 to day 75. In Stage 1 (Day 76–175), after both systems reached steady states (Day 101–143), despite the shorter SRT in the experimental system (i.e., 10 d *vs* 12 d in control), the average VS destruction (35.1 ± 1.5 %) in the experimental one was 16.2 % higher ($p < 0.01$) than that of the control one (30.2 ± 1.4 %) (Fig. 1A). A similar improvement of 13.2 % in TCOD removal was observed in the experimental system compared with the control one in Stage 1 (37.6 ± 1.9 *vs* 32.9 ± 1.4 %) (Fig. 1B). As VS is primarily responsible for the TCOD in WAS, the enhanced TCOD further supports the improved VS destruction observed in the experimental reactors.

After both systems reached stable in Stage 2 (Day 176–223), comparable average VS destruction ($p > 0.05$) was achieved between the experimental system (30.0 ± 1.4 %) and the control system (30.5 ± 1.7 %), although the experimental system had a shorter SRT (8 d *vs* 12 d in control). This was also supported by the comparable TCOD removal ($p > 0.05$) in both systems (33.1 ± 2.4 % in the experimental system and 34.2 ± 2.4 % in the control system). Therefore, FA pretreatment is effective in maintaining performance with a reduced HRT of 8d.

The VS destruction is also reflected by the TAN (NH_4^++NH_3-N) generation [22]. In comparison to the control system, the TAN concentration (Fig. 1C) increased by 20.5 % on average in stage 1 and reached comparable levels with control in stage 2 (externally added TAN by FA pretreatment was excluded). This observation was consistent with

Table 2
Performance of the experimental and control systems in Baseline 1, Stage 1 and Stage 2 (after both systems reached stable performance).

		Control system			Experimental system		
		Baseline	Stage 1	Stage 2	Baseline	Stage 1	Stage 2
Sludge destruction	VS destruction (%)	30.4 ± 1.9 % (n = 12)	30.2 ± 1.4 % (n = 13)	30.5 ± 1.7 % (n = 15)	31.0 ± 2.0 % (n = 12)	35.1 ± 1.5 % (n = 13)	30.0 ± 1.4 % (n = 15)
	TCOD removal (%)	33.7 ± 1.4 % (n = 12)	32.9 ± 1.4 % (n = 13)	34.2 ± 2.4 % (n = 15)	33.4 ± 2.2 % (n = 12)	37.6 ± 1.9 % (n = 13)	33.1 ± 2.4 % (n = 15)
	NH_4^++NH_3-N (mg/L)[a]	881 ± 40 (n = 13)	893 ± 50 (n = 12)	1026 ± 97 (n = 13)	871 ± 32 (n = 13)	1077 ± 64 (n = 12)	968 ± 123 (n = 13)
Methane production	Methane production (mL/g VS_{fed})	160 ± 5 (n = 38)	163 ± 8 (n = 44)	178 ± 8 (n = 48)	158 ± 6 (n = 38)	188 ± 9 (n = 44)	185 ± 8 (n = 48)
	Methane production (mL/day)	246 ± 13 (n = 30)	260 ± 18 (n = 44)	291 ± 19 (n = 48)	244 ± 17 (n = 30)	351 ± 25 (n = 44)	441 ± 7 (n = 48)
Stability	VFAs concentration (mg COD/L)	54 ± 16 (n = 6)	43 ± 12 (n = 7)	45 ± 12 (n = 10)	59 ± 18 (n = 6)	162 ± 22 (n = 7)	351 ± 51 (n = 10)
	pH	7.3 ± 0.1 (n = 12)	7.2 ± 0.1 (n = 25)	7.2 ± 0.1 (n = 20)	7.3 ± 0.1 (n = 25)	7.5 ± 0.2 (n = 25)	7.8 ± 0.2 (n = 20)
	Alkalinity (mg $CaCO_3$/L)	3418 ± 125 (n = 12)	3589 ± 176 (n = 12)	4117 ± 308 (n = 12)	3428 ± 180 (n = 12)	6300 ± 298 (n = 12)	5910 ± 449 (n = 12)
	VFA/alkalinity	0.02 ± 0.005 (n = 12)	0.01 ± 0.003 (n = 12)	0.01 ± 0.003 (n = 12)	0.02 ± 0.006 (n = 12)	0.03 ± 0.004 (n = 12)	0.06 ± 0.011 (n = 12)
	Biomass specific hydrolysis rate (g COD/g VS/day)	0.05 ± 0.01 (n = 3)	0.05 ± 0.01 (n = 3)	0.05 ± 0.01 (n = 3)	0.05 ± 0.01 (n = 3)	0.08 ± 0.01 (n = 3)	0.09 ± 0.01 (n = 3)
Pathogen and ARGs	Fecal Coliform level log MPN/g TS	/	4.2 ± 0.02 (n = 4)	3.7 ± 0.16 (n = 4)	/	2.8 ± 0.31 (n = 4)	3.0 ± 0.04 (n = 4)
	E. Coli level log MPN/g TS	/	4.0 ± 0.22 (n = 4)	3.7 ± 0.22 (n = 4)	/	2.0 ± 0.07 (n = 4)	2.5 ± 0.04 (n = 4)
	aac(6′)-Ib-cr (gene copies/g TS)	/	$2.8 \pm 0.4 \times 10^9$ (n = 3)	$2.9 \pm 0.2 \times 10^9$ (n = 3)	/	$2.0 \pm 0.5 \times 10^9$ (n = 3)	$2.7 \pm 0.1 \times 10^9$ (n = 3)
	blaTEM (gene copies/g TS)	/	$1.5 \pm 0.2 \times 10^7$ (n = 3)	$1.7 \pm 0.2 \times 10^7$ (n = 3)	/	$9.2 \pm 1.1 \times 10^6$ (n = 3)	$8.8 \pm 1.1 \times 10^6$ (n = 3)
	sul1 (gene copies/g TS)	/	$9.1 \pm 0.1 \times 10^8$ (n = 3)	$9.9 \pm 0.9 \times 10^8$ (n = 3)	/	$6.6 \pm 0.1 \times 10^8$ (n = 3)	$7.6 \pm 0.1 \times 10^8$ (n = 3)
	tetA (gene copies/g TS)	/	$5.8 \pm 0.5 \times 10^6$ (n = 3)	$3.3 \pm 0.4 \times 10^6$ (n = 3)	/	$3.1 \pm 0.3 \times 10^6$ (n = 3)	$2.0 \pm 0.4 \times 10^6$ (n = 3)
	tetX (gene copies/g TS)	/	$2.0 \pm 0.1 \times 10^7$ (n = 3)	$3.0 \pm 0.1 \times 10^7$ (n = 3)	/	$4.0 \pm 0.5 \times 10^6$ (n = 3)	$5.1 \pm 0.4 \times 10^6$ (n = 3)

a: added NH_4^+-N was excluded.

and further supports the increased and comparable VS destruction under shorter SRT due to the FA pretreatment.

3.2. Methane production

Consistent with the VS destruction, the FA pretreatment enabled an improved or comparable methane production per gram VS under shortened SRTs (Fig. 2A). In the baseline period, the control and the experimental systems reached convergence in the steady state with the average methane generation of 160 ± 5 and 158 ± 6 ml/g VS_{fed}, respectively (Table 2). With a shorter SRT in the experimental system, the average methane production was improved by 15.3 % in stage 1 (SRT = 10 d, 188 ± 9 ml/g VS_{fed} in the experimental system *vs* 163 ± 8 ml/g VS_{fed} in the control system). In stage 2, the average methane generation was 185 ± 8 ml/g VS_{fed} in the experimental system (SRT = 8 d), comparable to that in the control system (178 ± 8 ml/g VS_{fed}, SRT = 12 d). The enhanced (stage 1) and comparable (stage 2) methane production per gram VS with the control system further supported the VS destruction results in section 3.1, where the VS destruction increased by 16.2 % in stage 1 and reached comparable levels to the control in stage 2.

Furthermore, the average methane production of the experimental system increased by 35.0 % (from 260 ± 18 to 351 ± 25 ml/day) in the steady state of stage 1 and 51.5 % (from 291 ± 19 to 441 ± 27 ml/day) in the steady state of stage 2, compared with the control system (Fig. 2B and Table 2). This increment was also partially due to the increased organic loading rate (OLR, 20 % higher in stage 1 and 50 % higher in stage 2) under the shorter SRTs in the experimental system than the control system, besides the enhanced VS destruction (16 % higher in stage 1) due to the FA pretreatment.

3.3. Stability of the AD systems

The stability of both digestion systems was monitored by VFAs concentrations, pH and alkalinity [23,24]. In the baseline period, the VFAs concentrations in the experimental and control systems were similar ($p > 0.05$), which were 58.9 ± 18.0 and 54.4 ± 16.2 mg COD/L, respectively (Fig. 3A and Table 2). With the reduction of SRTs, temporal accumulation of VFAs was observed in the experimental reactor, where the peak VFAs concentrations reached 588.3 and 798.5 mg COD/L at first 3–10 days of stages 1 and 2, respectively. This is likely caused by the loading shock, which can be potentially alleviated by the real-world continuous feeding of pretreated sludge rather than daily feedings. After the transition, the experimental reactor gradually reached stable with VFAs concentrations at 161.8 ± 22.0 mg COD/L at steady period of stage 1 (SRT = 10 d) and 350.6 ± 50.7 mg COD/L at steady period of

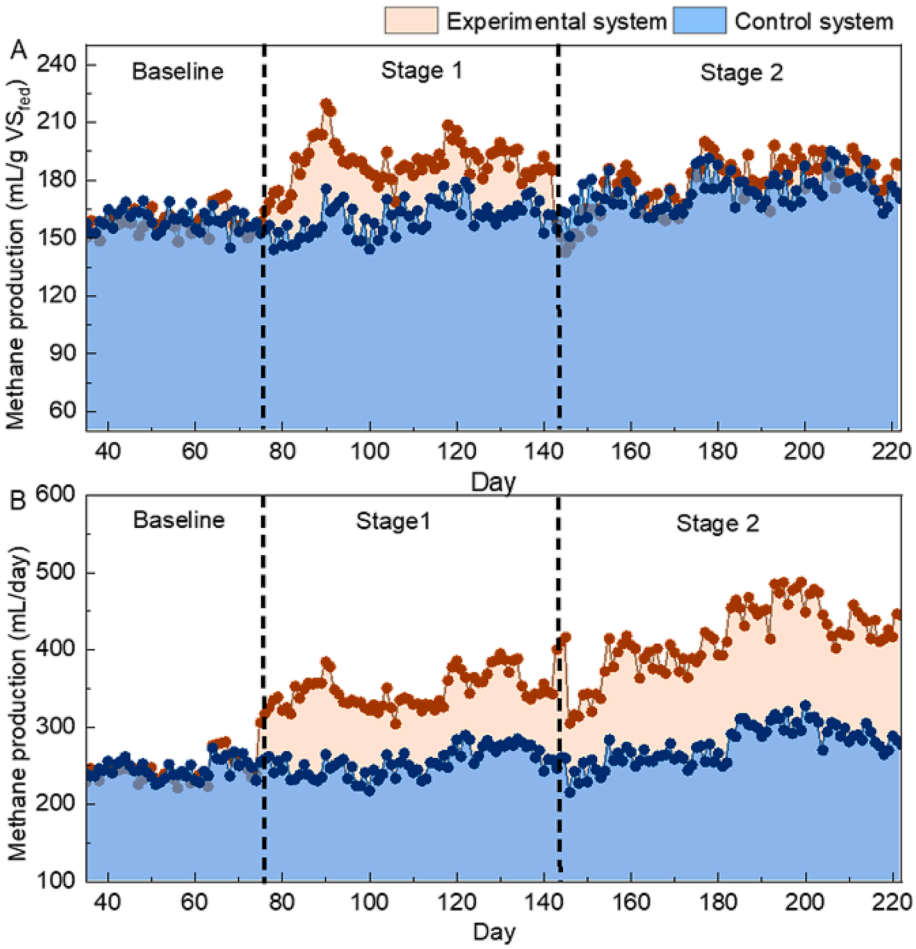

Fig. 2. Methane production: (A) Methane production according to VS; (B) Daily methane production in the two systems.

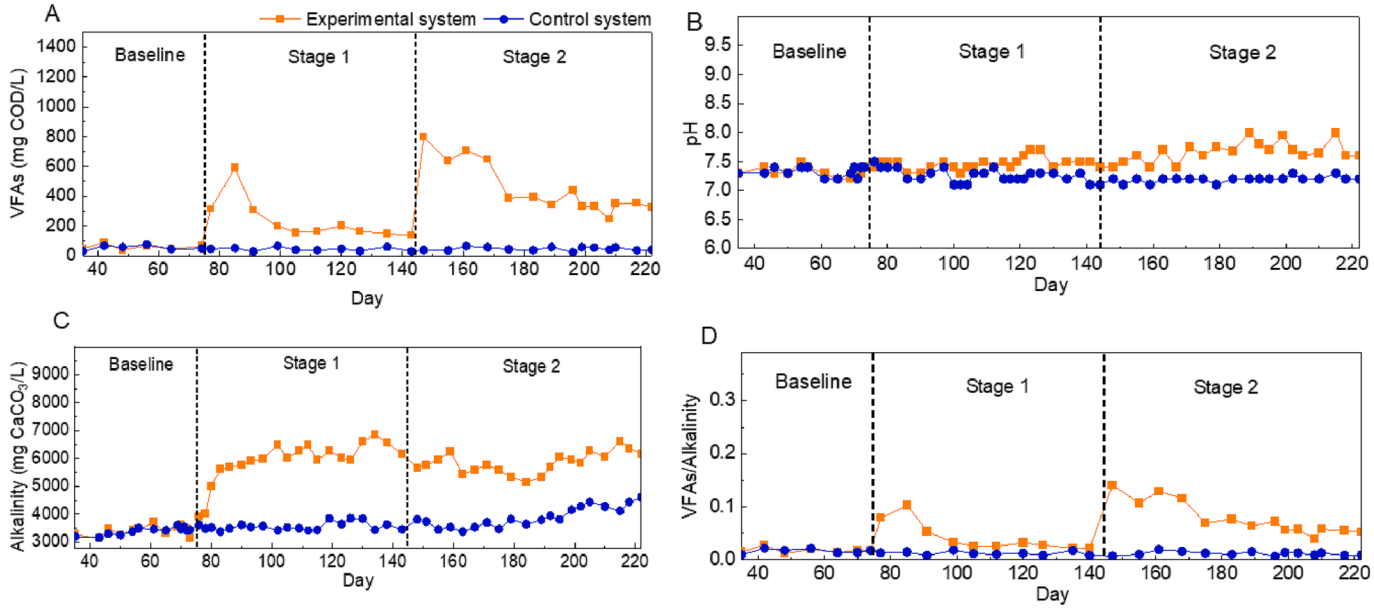

Fig. 3. VFAs concentrations, pH, alkalinity, and VFAs to the alkalinity ratio of the experimental and control systems in Baseline, stage 1 and stage 2.

stage 2 (SRT = 8 d) (Fig. 3A and Table 2). In comparison, the control system continuously maintained an almost constant VFAs concentration (42.7 – 54.4 mg COD/L). At the steady periods of baseline, stage 1 and stage 2, the VFAs of the control and experimental systems include acetic, propionic, butyric and valeric acids. The concentrations of different VFAs varied in different systems and stages (Fig. S2). However, the

percentages of main VFAs, i.e. acetic and propionic acids, between the two systems were similar, which account for > 60 % and > 30 %, respectively.

Although VFAs accumulated in the experimental system in stages 1 and 2, the pH over that period was 7.4–8.0, which was higher than pH in the control system (7.2 ± 0.1) (Fig. 3B). The higher pH was also indicated by the increased alkalinity in the experimental system, namely 6300 ± 298 in steady Stage 1 and 5910 ± 449 mg $CaCO_3$/L in steady stage 2, which are both higher than the control system (3428 ± 180 $CaCO_3$/L) (Fig. 3C). The increased pH and alkalinity (Fig. 3B &3C) are associated with the increased ammonia (Fig. 1C). The VFAs/alkalinity ratio under the shortened SRTs (shown in Fig. 3D) was between 0.03 and 0.06, within the normal range of stable AD operation (0.01 – 0.40) [15,25,26], indicating the stability of the digesters with FA pretreatment at shortened SRTs.

3.4. Biomass specific hydrolysis rate

Hydrolysis is commonly considered the rate-limiting step for particulate feed in AD systems [27,28]. In the Baseline stage, the biomass specific hydrolysis rates of the two systems were almost identical (0.05 ± 0.01 g COD/g VS/day, p> 0.05, Fig. 4). In stages 1 and 2, the biomass specific hydrolysis rate in the control system (SRT = 12 d) was around 0.05 g COD/g VS/d (Fig. 4). With the FA pretreatment, the biomass specific hydrolysis rate in the experimental system significantly increased by 60 % in stage 1 (0.08 ± 0.01 g COD/g VS/d, SRT = 10 d) and by 80 % in stage 2 (0.09 ± 0.01 g COD/g VS/d, SRT = 8d), in comparison to the control system (Fig. 4). The increased biomass specific hydrolysis rate is likely attributed to both the FA pretreatment and the decreased SRTs (increased OLR).

3.5. Microbial community

Due to the FA pretreatment and change of SRT, a clear microbial transition of bacteria was shown by the Beta diversity via PCoA (Fig. 5A). The bacterial community within the control system was comparable in all stages (Fig. 6A). Major changes in the bacterial microbial community from digested sludge samples was caused by the FA pretreatment as shown in PC1 (52.80 % of the variations) while the change of SRT from 10 d to 8 d slightly contributed to the variations in PC2 (21.61 %) (Fig. 5A). Although the phylum population displayed a slight variation in the experimental systems, the performance of the

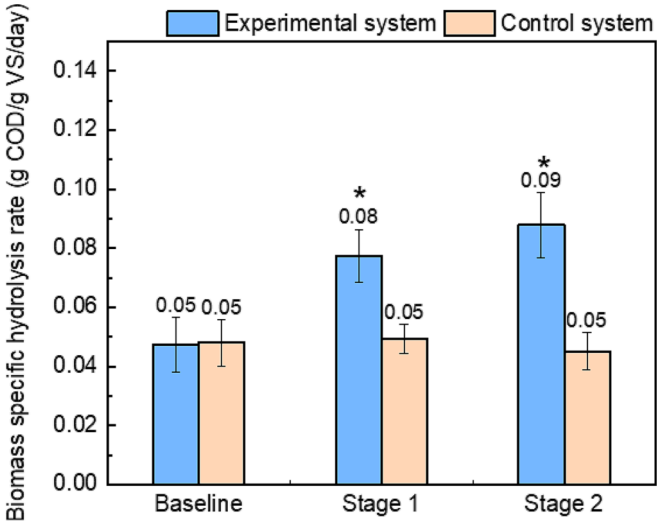

Fig. 4. Biomass specific hydrolysis rates of the two systems at the steady period of Baseline, stage 1 and stage 2. The error bar represents the standard deviation. Asterisks represent significant differences with p-values < 0.05.

experimental systems was stable (Section 3.1-3.3). In both stage 1 (SRT = 10 d) and stage 2 (SRT = 8 d), *Bacteroidota, Firmicutes, and Proteobacteria* were the dominant phylum, accounting for 55–76 % of the bacterial population in both systems. These main phyla are all related to the hydrolysis process, which could decompose polysaccharide, proteins and/or lipids [29–32]. Only slight bacterial community transitions between the hydrolysis related bacteria were observed in the experimental system (Fig. 6A). This was highly related to the respond of the microbe to stress caused by high TAN, VFAs concentration and pH in the experimental system [33], whereas the system could maintain normal performance. The experimental system was steady as shown in Section 3.3 and the ammonia inhibition was not observed.

Similarly, the FA pretreatment had a great influence on the archaeal community while shortened SRTs played a minor effect on the archaeal community (Fig. 5B). FA pretreatment majorly contributed to the archaeal community changes in PC1 (94.25 %), while SRT changes from 10 d to 8 d led to slight variation of archaeal community in PC2 (3.04 %). Overall, the relative abundance of acetoclastic methanogens (i.e. *Methanosaeta* and *Bathyarchaeia*) in the control and experimental systems were comparable, which were 30 –32 % and 30 – 31 %, respectively (Fig. 6B). Obviously, the acetoclastic methanogens remained one of the dominant methanogens rather than shifting to hydrogen utilizers, supporting that ammonia inhibition did not happen in the experimental system. The microbial shift from acetoclastic methanogens to hydrogenotrophic methanogens could be the signal of ammonia inhibition in AD system since acetoclastic methanogens are more sensitive to a high level of ammonia compared to hydrogenotrophic methanogens [34]. The total relative abundance of hydrogenotrophic methanogens, including *Candidatus Methanofastidiosum*, *Methanoculleus*, *Methanobrevibacter, RumEn_M2*, *Methanomassiliicoccus*, *Methanocorpusculum*, *Methanobacterium*, and *Methanosarcina*, was similar in the experimental system (69 – 72 %) and in the control system (68 %) (Fig. 6B). The relative abundances of some hydrogenotrophic methanogens, such as *Candidatus Methanofastidiosum* and *Methanobrevibacter*, decreased in the experimental system, whereas the relative abundances of *Methanoculleus and RumEn_M2* increased, compared to those in the control one. These internal community shifts among hydrogenotrophic methanogens in the experimental system should be caused by the potential selection pressure of FA over some hydrogenotrophic methanogens, which did not affect the hydrolysis process of the experimental AD system. Therefore, the comparable archaea community structure of the experimental and control systems supported the stable performance of the experimental system, which proved the feasibility of expanding sludge treatment capacity through FA pretreatment.

3.6. Pathogen removal

Fig. 7 presents the biomass-specific MPN of two typical pathogen indicators, Fecal Coliform and *E. Coli*, in both control and experimental systems during steady stages 1 and 2. In the control system, AD reduced the Fecal Coliform by 0.8 –1.2 log MPN/g TS and *E. Coli* by 0.9 log MPN/g TS (hereafter referred to as 'log' in the following text) in stages 1 and 2. For the experimental system, FA pretreatment alone reduced Fecal Coliform and *E. Coli* by 1.4 – 1.7 and 1.1 – 1.3 log, respectively, in stages 1 and 2 (Fig. 7). The combined FA pretreatment and AD achieved comparable removals of Fecal Coliform and *E. Coli* in stages 1 and 2, which are 2.1 – 2.6 and 2.3 – 2.9 log in stage 1 (SRT = 10 d) and in stage 2 (SRT = 8 d), respectively. In comparison to the control system, despite the shorter SRTs in the experimental system, Fecal Coliform removal and *E. Coli* removal were enhanced by 1.3 – 1.4 log and 1.4 – 2.0 log, respectively. This result implies that FA pretreatment is valuable in promoting pathogen removal even at a shorter SRT of 8 d in AD.

Furthermore, the Fecal Coliform and *E. Coli* in digested sludge from the experimental system were 2.8 – 3.0 log and 2.0 – 2.5 log, respectively. The FA pretreatment enables digested sludge in stage 1 to meet the requirement for Grade A biosolids according to the NSW

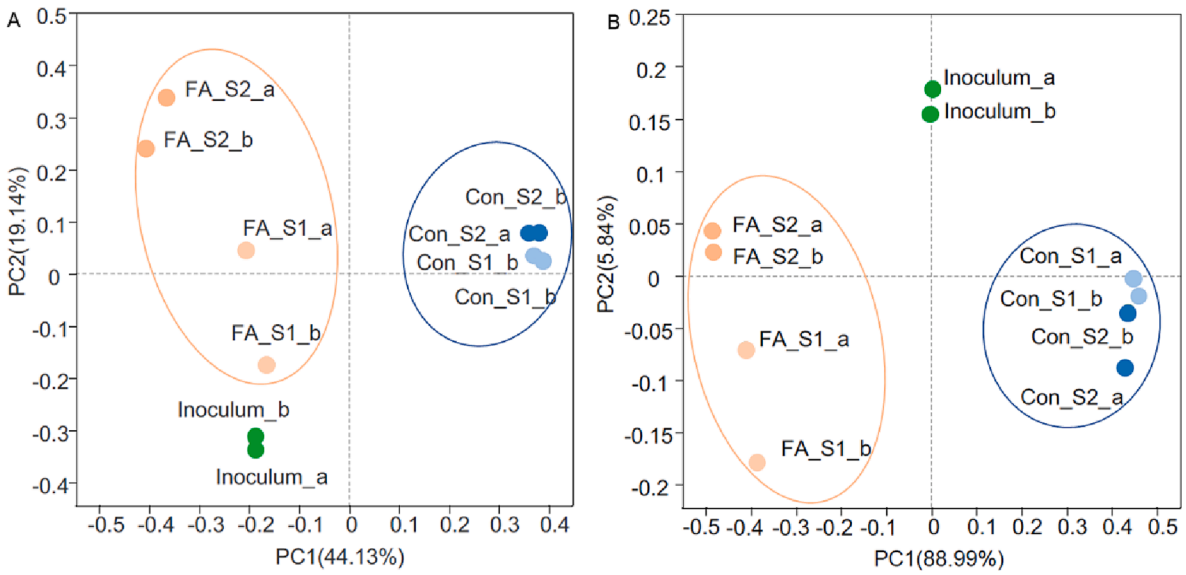

Fig. 5. PCoA analysis for the bacterial communities of inoculum and digestates from systems at stages 1 and 2 (A); PCoA analysis for the archaeal communities of the inoculum and digestates from systems at stages 1 and 2 (B). The PCoA analyses for samples were plotted in duplicates (in the same colour). FA and Con indicate the experimental and control system, respectively. S1 and S2 indicate stage 1 (10 d SRT in the experimental; 12 d SRT in the control) and stage 2 (8 d SRT in the experimental; 12 d SRT in the control). The "a" and "b" are duplicated for each other.

Environmental Protection Agency, Australia ((EPA) 2000 [35]. This suggests quality of biosolids can be improved and potentially reused as safe biosolids in real-life applications.

3.7. ARGs removal

The absolute abundances of ARGs in the feed WAS and digested sludge with and without FA pretreatment were investigated to assess the influence of FA pretreatment at shortened SRTs on the removals of ARGs. The absolute abundances of *aac(6′)-Ib-cr*, *blaTEM*, *sul1*, *tetA* and *tetX* are from 2.0×10^6 to 3.8×10^9 gene copies/g-TS in all the sludge samples (i.e. WAS and digested sludge in stages 1 and 2 from the control and experimental systems) (Fig. 8A), which were in line with the previous study [16].

In the control system, the removal extent of ARGs was comparable in stages 1 and 2 ($p > 0.05$), where the AD reduced the absolute abundance of *aac(6′)-Ib-cr*, *sul1*, *tetA* and *tetX*, by 22 – 23 %, 29 – 30 %, 90 – 92 % and 68 – 75 %, but increased the absolute abundance of *blaTEM* by 41 – 66 %. In the experimental system with FA treatment, the removal extent of ARGs was also comparable in stage 1 and stage 2 ($p > 0.05$). The absolute abundance of *aac(6′)-Ib-cr*, *blaTEM*, *sul1*, *tetA* and *tetX* was reduced by 28 – 44 %, 12 – 13 %, 46 – 49 %, 95 % and 94 – 95 %. In comparison to the control system, despite the shortened SRT, FA pretreatment achieved similar removals of *aac(6′)-Ib-cr* (28 – 44 %) and *tet A* (95 %) and significantly improved the removal of *sul1* and *tetX* ($p < 0.05$) by 53 – 67 % and 27 – 40 %, respectively. FA pretreatment also removed the *blaTEM* by 12–13 %, whereas *blaTEM* increased in the control system. (Fig. 8B). Therefore, FA pretreatment is able to improve the removal of *sul1*, *tetX* and *blaTEM* while implemented for expanding the digester capacity. Overall, FA pretreatment promoted the removal of targeted ARGs by 34–86 %.

4. Discussion

This study for the first time demonstrated the feasibility of using FA pretreatment to expand the treatment capacity of anaerobic digesters while bringing additional benefits. Although FA pretreatment has been reported to improve the performance of an anaerobic digester, its application in upgrading the anaerobic digester for treatment capacity expanding is still largely unknown. This was experimentally demonstrated through over 7 months long-term tests in two systems without and with FA pretreatment (at shortened SRTs). The results demonstrated that FA pretreatment can expand the treatment capacity of an anaerobic digester by up to 50 %, supported by the results of VS destruction, TCOD removal, and methane production per gram VS.

Instead of replacing a larger digester or adding an additional digester, which requires additional space and ancillary equipment, FA pretreatment only requires a small mixing tank (SRT = 1 day) before the anaerobic digester. Specifically, an additional digester would occupy 7–10 times more space than the mixing tank for FA pretreatment, incurring higher costs. Thus, FA pretreatment is a promising technology for expanding the treatment capacity of existing digesters to accommodate the increased amount of sludge.

The expanded capacity can be attributed to the increased biomass specific hydrolysis rate. Hydrolysis was considered the rate-limiting process in AD [11]. In this study, FA pretreatment at shortened SRTs increased the biomass specific hydrolysis rates of the experimental system by 60 % and 80 % at the SRTs of 10 d and 8 d (0.08 ± 0.01 and 0.09 ± 0.01 g COD/g VS/d), respectively, compared with the control system without pretreatment at the SRT of 12 d (0.05 ± 0.01 g COD/g VS/d). This is consistent with our previous observations, where FA causes cell lysis and EPS destruction, which improved the hydrolysis rate in the batch BMP tests and increased the solubilization [15].

Thermal hydrolysis and free nitrous acid (FNA) pretreatments have been previously used to expand the capacity of the anaerobic digester. Thermal hydrolysis pretreatment (140–160 °C) increases the capacity of the anaerobic digester by 33–50 % [36,37], which is comparable with FA pretreatment. However, thermal hydrolysis requires energy input and significant capital investment. FNA pretreatment doubled the digester capacity with a further 30–40 % increase in VS destruction [38], which is higher than FA pretreatment. However, FNA pretreatment requires an extra side-stream nitritation reactor to produce FNA on-site [39] and the nitritation reactor does not exist in most WWTPs. This limits the applicability of the FNA pretreatment. In addition, FNA pretreatment fails to improve pathogen removal when the digester treatment capacity was expanded, whereas FA pretreatment does. However, it should be noted that the above comparison should be considered as indicative only because the conditions in different studies were different and these conditions (e.g. sludge origins) would affect the reactor performance.

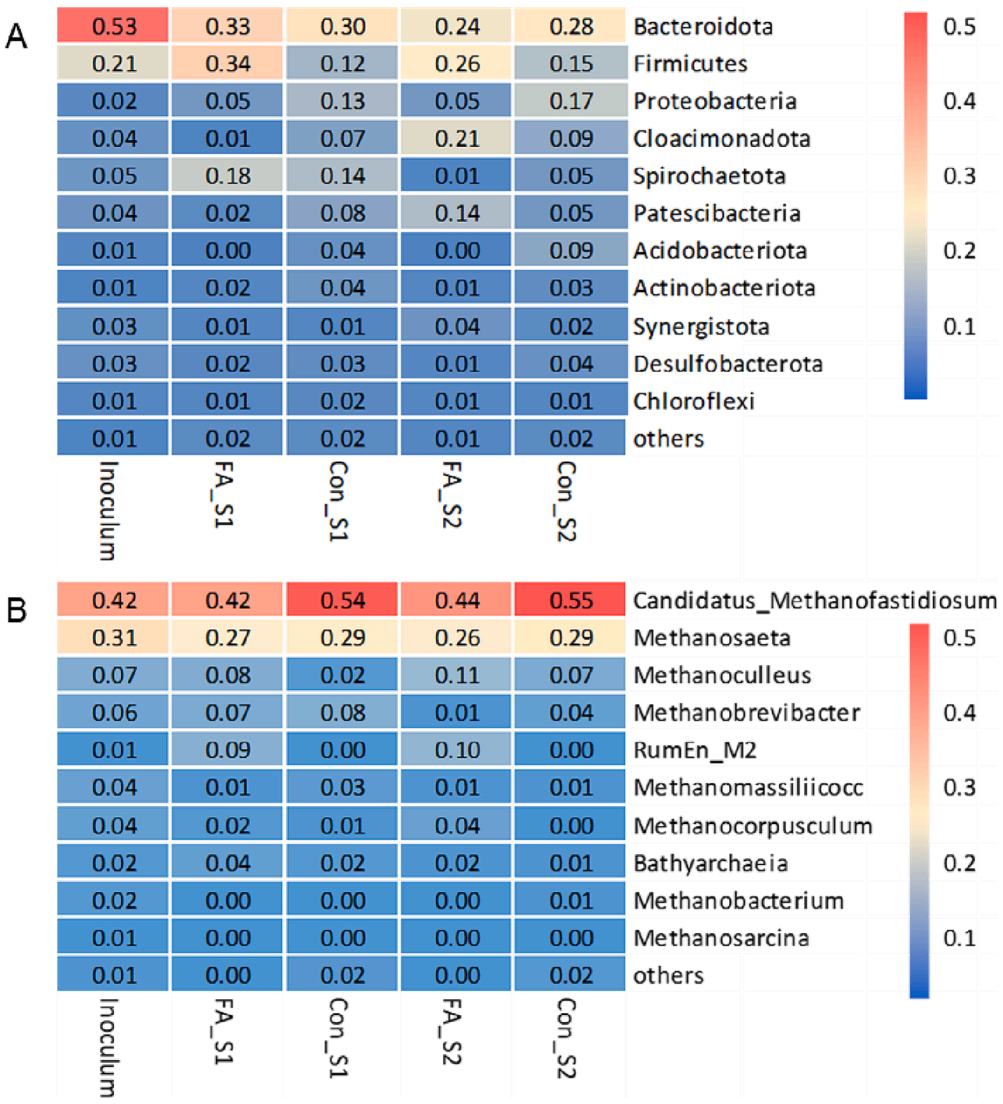

Fig. 6. Heatmap shows the (>1%) relative abundance of bacteria at the phylum level. The phylum accounting for<1 % in the samples are classified as "the others" (A); Heatmap shows the populations (>1%) relative abundance of archaea at the genus level. The genus accounting for<1 % in the samples are classified as "the others" (B).

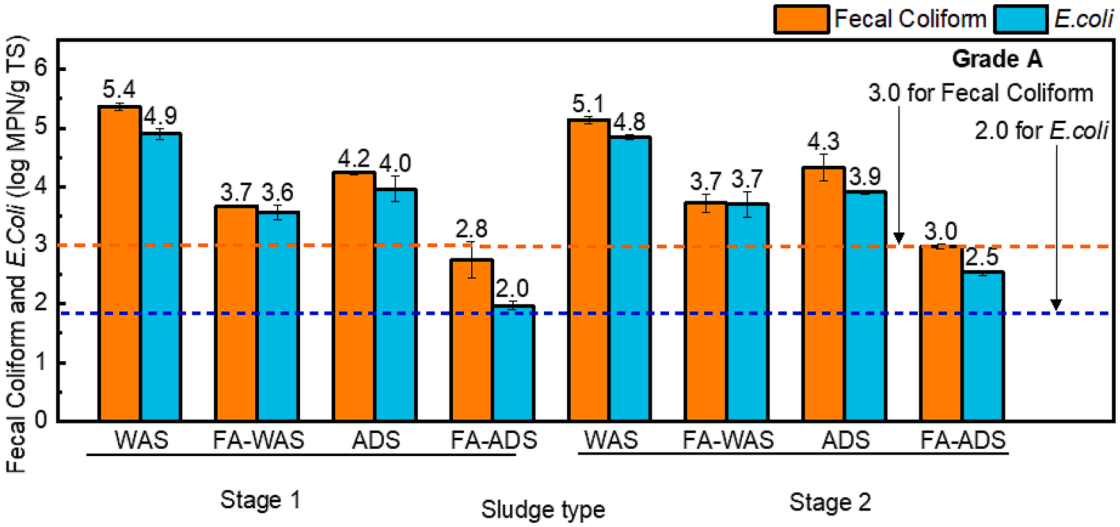

Fig. 7. Fecal Coliform and *E. Coli* concentrations in different sludges in two experimental stages (Stage 1 and Stage 2). (WAS: waste activated sludge; FA-WAS: FA treated WAS; ADS: anaerobically digested sludge; FA-ADS: ADS with FA pretreatment). The error bar indicates the standard deviation.

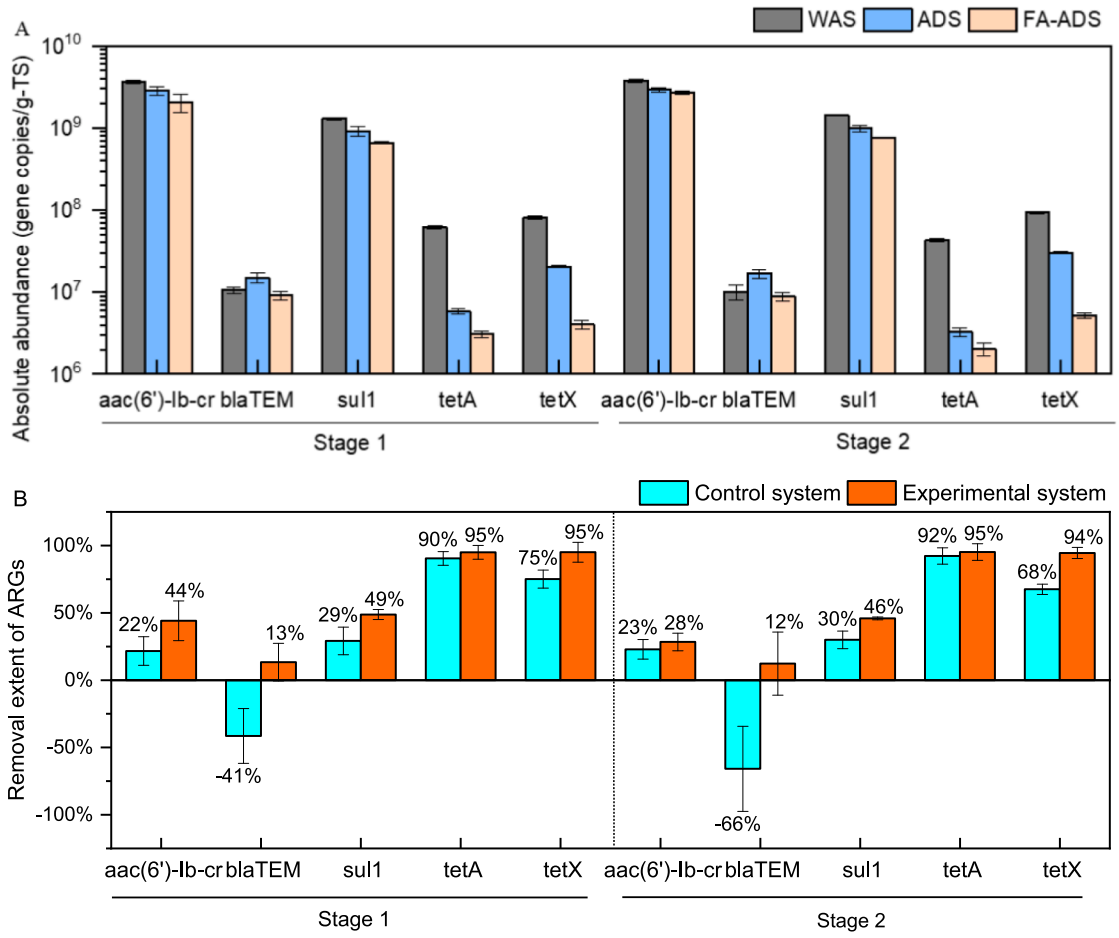

Fig. 8. Absolute abundances of ARGs in WAS, ADS and FA-ADS at stages 1 and 2. (WAS: waste activated sludge; ADS: anaerobically digested sludge; FA-ADS: ADS with FA pretreatment (A). Removal extent (%) of ARGs in ADS with and without FA pretreatment compared to raw WAS at stages 1 and 2. Negative values indicate increases in the target gene (B). The error bar indicates the standard deviation.

FA pretreatment at shortened SRTs raised the acetic acid concentrations in the digester (Fig. S2). In the steady period of stages 1 and 2, the acetic acid concentrations were 107 ± 15 and 218 ± 32 mg COD/L, respectively, which was higher than those in the control system (25 – 35 mg COD/L) (Fig. S2). Considering the increased biomass specific hydrolysis rate in the experimental reactor in stages 1 and 2, the increased acetic acid concentration (Fig. S2) suggests that methanogenesis instead of hydrolysis may be the limited step in experimental reactors with FA pretreatment. This is also supported by the comparable relative abundance of acetoclastic methanogens between the experimental and control systems.

The 'refractory' SCOD in the digestion liquor increased by 1118 and 724 mg/L with FA pretreatment under an SRT of 10 d and 8 d, respectively (Fig. S1), compared to the control system. As the digestion liquor is usually returned to the wastewater treatment process of the WWTPs, the increased 'refractory' SCOD in the digestion liquor would rise the COD concentration in the effluent of the WWTPs. Considering that the volume of digestion liquor in WWTPs is generally around 1 % of the influent [40], the SCOD increase in the effluent of the WWTPs is estimated to be around 11 and 7 mg/L with FA pretreatment under an SRT of 10 d and 8 d, respectively. Such an increase in the effluent COD has been commonly observed with other pretreatment approaches. For instance, the commercialized thermal pretreatment led to the SCOD increase of 12–15 mg/L in the effluent [19].

Despite the shortened SRT in the experimental system, FA pretreatment showed efficient pathogen removal compared with control. Also, the change of SRT from 10 d to 8 d with FA pretreatment showed negligible impacts ($p > 0.05$) on the pathogen removal in the experimental system, implying that the lower pathogen levels in the experimental reactors are likely attributed to the FA pretreatment. This was also observed in our previous finding [15]. The lower level of pathogens in the ADS with FA pretreatment indicated a higher quality of sludge (biosolids), which can be potentially applied to agriculture, forestry, and urban landscaping with minimized risk. Therefore, FA pretreatment not only expands the digester capacity but also brings additional environmental benefits for sludge reuse and disposal.

Apart from pathogens, the potential risk associated with ARGs diffusion and transfer into the regional land during sludge reuse or disposal is of high concern [41]. This study indicated that FA pretreatment promoted the removal of *aac(6′)-Ib-cr, sul1*, *tetX* and *blaTEM*, and did not affect the removal of *tetA* while implemented for expanding the digester capacity (i.e. at shortened SRTs) compared with the control system. The enhanced ARGs removal due to the FA pretreatment was potentially related to: 1) FA pretreatment kills the host of ARGs (i.e. antibiotic resistant bacteria) as FA could diffuse the cell membrane [13]; 2) FA could induce DNA damage, particularly in cell-free ARGs [42]; 3) The stressful circumstance in experimental system, including high pH and TAN concentration, may also cause the oxidation stress for microorganisms, which may lead to the membranolysis of these ARGs host (microorganisms) [43]. The reduction of ARGs host would then reduce the ARGs concentration. The enhanced ARGs removal will mitigate the risk of sludge reuse to the environment.

5. Conclusion

To date, intensive studies focused on FA pretreatment application in energy recovery and high-value products generation from sludge. However, in this study, FA pretreatment is proposed and experimentally evaluated for the first time to expand the treatment capacity of an anaerobic digester and meanwhile to eliminate the pathogen and ARGs in sludge (biosolids). FA pretreatment expanded the capacity of the anaerobic digester by up to 50 % with comparable VS destruction and methane production per VS to the control system without FA pretreatment under a common SRT. This provides an urgently-needed and practical resolution to upgrade the capacity of existing anaerobic digester with minimized financial expenditure and operational disruptions. The feasibility and stability of an enlarged AD system applied FA pretreatment with shortened SRTs has been supported by the improved biomass specific hydrolysis rates (by 60–80 %) and also verified by the similar microbial community structure of the experimental system and the control system. Additionally, FA pretreatment brought additional benefits in the removal of pathogens and ARGs even under shortened SRTs. The removal of Fecal Coliform and *E. Coli* was enhanced by 1.3–2.0 log MPN/g TS, meanwhile the removal of ARGs was also improved by 34–86 %. These improvements reduce the environmental risks due to sludge reuse and disposal. Although FA pretreatment implementation requires the pilot and full-scale assessment in the future, this study provides the preliminary assessment and promotes the overall technology benefits of FA pretreatment.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2023.142846.

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