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Acetic acid effects on methanogens in the second stage of a two-stage anaerobic system

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Abstract

This study reports on biomass tolerance towards high concentrations of acetic acid (HAc) within the system. Biomass from the second stage of a two-stage anaerobic sludge digestion system was used for this study. Microbial community analysis by 454 pyrosequencing highlighted hydrogenotrophic *Methanomicrobiales* was the predominant archaeal population in the second stage (> 99% of the total archaeal community). Second stage biomass degraded HAc up to 4200 mg HAc L^{-1} without observable lag phase. However, at HAc-shock loading of 7400 mg HAc L^{-1}, it showed a one day lag phase associated with decreased biomass activity. After stepwise HAc-acclimation over 27 d, the biomass degraded HAc of up to 8200 mg HAc L^{-1} without observable lag phase. The dominance of *Methanomicrobiales* had

remained unchanged in proportion - while the total archaeal population increased during acclimation. This study showed stepwise acclimation could be an approach to accommodate HAc accumulation and hence higher concentrations resulting from an enhanced first stage.

Keywords

Two-stage anaerobic system; acetic acid inhibition; acclimation; hydrogenotrophic methanogens; pyrosequencing; biomass activity.

List of abbreviations

A	The acclimated biomass
AM	Aceticlastic methanogens
AMPTS	Automatic methane potential test system
ATP	Adenosine triphosphate
CSTR	Continuous stirred tank reactor
day	d
FID	Flame ionization detector
$\Delta G^{0'}$	the Gibbs free energy under standard condition
GC	Gas chromatography
HAc	Acetic acid
HM	Hydrogenotrophic methanogens
HRT	Hydraulic retention time
k	HAc degradation rate constant
MBT	*Methanobacteriales*
MMB	*Methanomicrobiales*
MSC	*Methanosarcinaceae*

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MST	*Methanosaetaceae*
N.A.	Not available
OTUs	Operational taxonomic units
PBS	Phosphate buffered saline
ppt	parts per thousand
qPCR	Quantitative polymerase chain reaction
R^2	Regression coefficient
S	The unacclimated biomass
SAOB	Syntrophic acetate-oxidizing bacteria
TS	Total solids
VFAs	Volatile fatty acids
VS	Volatile solids
vs.	versus
VSS	Volatile suspended solids

1. Introduction

The anaerobic process has been considered a cost-efficient and sustainable approach for high strength wastewater treatment and energy recovery. The process comprises four steps - hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Li et al., 2012). The two-stage anaerobic system has drawn attention due to its tolerance to higher organic loading rates, higher hydrolysis and acidogenesis efficiencies coupled with increased energy recovery over the single-stage anaerobic system (Nasr et al., 2012). In order to enhance the efficiency of the two-stage anaerobic system, many studies have focused on optimizing hydrolysis and acidogenesis reactions in the first stage to maximize the substrate availability for methanogens in the second stage (Dugba and Zhang, 1999; Nielsen et al., 2004;

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Aslanzadeh et al., 2014). However, improved hydrolysis and acidogenesis efficiencies in the first stage could lead to accumulation of volatile fatty acids (VFAs), which are potential inhibitors for methanogens at high concentrations. Until now, less attention has been paid to examine the effects of hydrolyzed and fermented substances (e.g. VFAs) generated in the first stage on methanogenic activities in the second stage. During hydrolysis and acidogenesis, acetic acid (HAc) is one of the major VFAs products, and it serves as a precursor for two-thirds of methane generation (Rincón et al., 2008). However, high HAc concentration could inhibit methanogenic activity if it exceeded the inhibition threshold. The study by Fukuzaki et al. (1990a) was among the first few that investigated HAc effects on methanogens in single-stage batch tests, and their results indicated the concentration of undissociated acetic acid was the key factor affecting methanogenesis from HAc. Mawson et al. (1991) also reported that increasing HAc concentration from 400 to 2500 mg L^{-1} would reduce HAc utilization rate in single-stage batch tests. More recently, Xiao et al. (2013) studied the inhibitory effects of HAc on methanogens in a two-phase anaerobic process, and different HAc effects on methanogens in the acid and methane phases were observed. To date, there is still limited information on methanogens' tolerance to high HAc concentrations in the second stage of a two-stage anaerobic system.

Lins et al. (2012) demonstrated acclimation feasibility to alleviate HAc accumulation during the start-up of a single-stage fed-batch reactor, however, its feasibility to alleviate HAc accumulation in the second stage of a two-stage system was not reported. It has been noted the methanogen community in the second stage of the two-stage anaerobic system would be different from the single-stage system (Merlino et al., 2013). Hence, it would be necessary to also investigate the possibility of acclimating biomass in the second stage to alleviate HAc accumulation. Such

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investigation could also shed light on the underlying microbial shift and biomass activity associated with HAc acclimation.

This study focused on HAc effects on biomass from the second stage of a two-stage anaerobic system. Studies were carried out to determine the effects of HAc-acclimation on methanogenic activities. The related biomass activity was investigated as well to understand the acclimation process. Pyrosequencing and quantitative polymerase chain reaction (qPCR) were employed to characterize the methanogen profiles in the second stage, and during HAc-shock loading and acclimation. Insights gained from this study were expected to provide guidance on maintaining second stage stability as the first stage became more effective at hydrolysis and acidogenesis.

2. Materials and methods

2.1. Two-stage reactor set-up

The two-stage continuous stirred tank reactor (CSTR) anaerobic system was operated for 121 d. It had a 7.5 L first-stage reactor and a 42.5 L second-stage reactor. The first stage was operated at 55 ^{0}C, pH 8.5 $\pm$ 0.3 and hydraulic retention time (HRT) of 3 d, and the second stage was operated at 35 ^{0}C, pH 7.1 $\pm$ 0.2 and HRT of 17 d. Seed sludge was collected from an anaerobic sludge digester at a local waste reclamation plant. Feed sludge (pH 5.7 - 6), containing 25 $\pm$ 2 g total solids (TS) L^{-1} and 20 $\pm$ 2 g volatile solids (VS) L^{-1}, was collected at regular intervals from the same plant and stored in 4 ^{0}C cold room before use. Throughout the 121 d of reactor operation, the accumulated VFAs distribution in the first stage followed the order of HAc (49%) > iso-valeric acid (15%) > propionic acid (14%) > iso-butyric acid (7%) > n-butyric acid (4%). In the second stage, there was no residual VFA. The average

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HAc concentrations in the feed sludge, first and second stages were 576, 2782 and 0 mg HAc L^{-1}, respectively. The term acetic acid (HAc) used throughout this study is to indicate the chemical species in all its forms (generic form), including dissociated acetic acid and undissociated acetic acid. During the 121 d operation, sludge was sampled at intervals from all reactors for salinity, volatile suspended solids (VSS) and microbial tests.

2.2. Experimental design

2.2.1. HAc effects on biomass during HAc-shock loading

The experiments to investigate HAc effects on biomass from the second stage of the aforementioned two-stage anaerobic system were carried out in 5 L fermentors (BIOSTAT Aplus MO, Satorius, Germany) in batch configuration. 2 L biomass (VSS =10.63 g L^{-1}) drawn from the aforementioned second stage was used as inoculum with nitrogen sparged during the transfer process. From preliminary laboratory experiments using automatic methane potential test systems (AMPTS) (Bioprocess Control Sweden AB, Sweden) (Supplementary data: Fig. S1), the biomass showed highest activity while incubated with initial HAc concentration of 2000 mg HAc L^{-1} compared to other concentrations. Therefore, the baseline HAc concentration tested was 2100 mg HAc L^{-1} in this study. 2 L synthetic medium (Labib et al., 1992) containing HAc was fed into the fermenters for initial HAc concentrations of 2100, 4200, 6400, and 7400 mg HAc L^{-1} in the mixed liquor, respectively. Various incubation periods were maintained for these batch tests in anticipation of HAc degradation rates which might result. The test groups were designated as 2100S, 4200S, 6400S, and 7400S where "S" denotes shock-loading/unacclimated biomass (Table 1). The fermentor system was controlled at pH of 7.0 ±0.2 with 2.5 N HCl and 2.5N NaOH. Temperature was set at 35 ^{0}C with continuous speed at 200 rpm. Sludge

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samples were withdrawn for VFAs at predetermined intervals on a daily basis. Sludge samples for both ATP and VSS tests were withdrawn at 0^{th} d and 1^{st} d. Sludge for microbial tests (pyrosequencing and qPCR) and related VSS test were sampled at the end after the depletion of each added HAc concentration.

2.2.2. Acclimation of biomass

Similarly, experiments to investigate biomass acclimation to HAc were performed in 5 L fermenters (BIOSTAT Aplus MO, Satorius, Germany) in batch configuration. 2 L biomass (VSS =10.63 g L^{-1}) drawn from the second stage was utilized as inoculum. For feed substrate, the baseline HAc concentration for the acclimation experiment was set at 2100 mg HAc L^{-1} on day (d) 0. Additional HAc was gradually fed into the fermentor on the 4^{th}, 8^{th}, 13^{th}, 18^{th}, and 23^{th} d to achieve HAc concentrations of 3700, 4100, 6000, and 8200 mg HAc L^{-1} in the mixed liquor, respectively. The time at which each HAc increment was dosed would depend on when the previous HAc dose was depleted. For example, after the depletion of 2100 mg HAc L^{-1}, the mixed liquor was centrifuged (12857×*g*, 10 min) and resuspended with 1×phosphate buffered saline (PBS) solution (pH=7.4) to make a total volume of 2 L. This was to avoid salinity accumulation during pH adjustment since it might mask HAc inhibition (Li et al., 2012). Another 2 L nutrient medium (Labib et al., 1992) containing 3700 mg HAc L^{-1} was then fed into the fermentor on the 8^{th} d. A similar procedure was adopted with HAc concentrations of 4100, 6000, and 8200 mg HAc L^{-1}. The test groups were designated as 2100A, 3700A, 4100A, 6000A, and 8200A where "A" denotes acclimated biomass (Table 1). The system was controlled at pH of 7.0 ± 0.2, temperature of 35 ^{0}C and stirrer's mixing speed at 200 rpm. Sludge samples were withdrawn for VFAs at predetermined intervals on a daily basis. Sludge

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175 samples for both ATP and VSS tests were withdrawn at 0^{th} d and 1^{st} d. Sludge for
176 microbial tests (pyrosequencing and qPCR) and related VSS test were sampled at the
177 end after the depletion of each added HAc concentration.
178
179 *2.3. Physico-chemical analysis*
180 VFAs were analyzed with gas chromatography (GC) and a flame ionization
181 detector (FID) (Agilent Technologies Inc., USA). The sludge sample was centrifuged
182 at 12857 × *g* for 5 min and the supernatant filtered through a 0.2 μm nylon membrane
183 filter. The filtered sample with 10% formic acid was added into a 1 mL vial at a
184 ratio of 9:1 (v : v). A detailed description of VFAs analysis can be found in (Xiao et
185 al., 2013). The calculation of HAc concentration is through multiplying molecular
186 weight (60.05 g mol^{-1}) by measured HAc mole number from GC.
187 For salinity measurement, the sludge sample was centrifuged at 5143 × *g* for 5
188 min, and then was filtered through 0.45 μm sterilized nylon membrane filter
189 (Whatman, UK) (Walton, 1989). Salinity was measured with a conductivity meter
190 (Mettler-Toledo Ltd., Singapore).
191 ATP measurement had been used as a rapid and simple method for evaluating
192 total biomass activity (Shanmugam and Horan, 2009). The amount of ATP present in
193 the biomass would be proportional to the luminescence released from the reaction of
194 biomass with the reagent BacTiter-Glo™ (Promega, USA). Dilution was made with 1
195 × PBS buffer (pH 7.4) to ensure the samples could be interpolated within the standard
196 curve. 100 μL specific commercial reagent and 100 μL standard or sludge samples
197 were incubated on opaque-walled 96-well plates on an orbital shaker for 5 min. The
198 luminescence data was recorded with a plate reader (Infinite M200Pro Tecan, SciMed,
199 USA). The specific ATP concentration was normalized against VSS concentration

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200 (Hwang and Hansen, 1998; Chen, 2000).

201 VSS tests were in accordance with standard methods (APHA, 2005).

202

203 *2.4. Microbial community analysis*

204 *2.4.1. 454 high-throughput 16S rRNA gene pyrosequencing*

205 Total genomic DNA was extracted from samples using a powersoil DNA
206 isolation kit (Mobio laboratories, USA). The 16S rRNA gene universal bacterial
207 primer set of 28 F and 519 R, targeting V1-V3 hypervariable region (Islam et al.,
208 2015) and 16S rRNA gene universal archaeal primer set of 517 F and 909 R, targeting
209 V4 - V5 hypervariable region (Wang and Qian, 2009) were used for amplification.
210 The pyrosequencing analysis of DNA samples was conducted by Research and
211 Testing Laboratory (Texas,USA), with details as described by Xiao et al. (2014).

212

213 *2.4.2. Real-time quantitative PCR*

214 DNA was extracted from the sludge sample with an automatic nucleic acid extractor
215 (MagNA Pure) in accordance with the manufacturer's protocol (Roche Diagnostics
216 GmbH, Germany). Total archaeal population was quantified with primers (787F and
217 1059R) and probes (915F) described by Lee et al. (2009) and Shin et al. (2010). qPCR
218 was conducted using LightCycler 480 (Roche Diagnostics GmBH, Germany). A 20
219 μL reaction mixture was prepared in accordance with the protocol of LightCycler 480
220 Probes Master kit (Roche, Germany). The two-step thermal cycling procedure was
221 performed as follows: 1) 10 min denaturation at 95 ^{0}C, followed by 10 s denaturation
222 (55 cycles) at 95 ^{0}C; 2) 30 s combined annealing and elongation at 60 ^{0}C, and 10 s
223 cooling at 40 ^{0}C. The description for standard curves using representative strains can
224 be found in Kim et al. (2013).

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3. Results and discussion

3.1. HAc degradation during HAc-shock loading and acclimation

The biomass was subjected to HAc-shock loading concentrations at 2100, 4200, 6400 and 7400 mg HAc L^{-1}. Fig. 1 shows there was a one day lag phase for HAc degradation at shock loading concentrations of 6400 and 7400 mg HAc L^{-1} (Fig. 1c and Fig. 1d). There was, however, no observable lag phase for the unacclimated biomass to degrade HAc concentrations at 2100 and 4200 mg HAc L^{-1} during the first day of incubation (Fig. 1a and 1b), with HAc completely depleted by 4 d and 6 d, respectively. It was noted the HAc inhibition threshold in this study was higher compared to the typically reported values (2000 - 3000 mg HAc L^{-1}) (Mawson et al., 1991; Xiao et al., 2013). This may be related to the pre-exposure and acclimation of biomass to high HAc concentration of 2782 mg L^{-1} in the first stage.

Stepwise acclimation was performed by gradually increasing HAc concentrations from 2100 mg HAc L^{-1} untill 8200 mg HAc L^{-1} over 27 d. With such stepwise acclimation, there was no observable lag phase in HAc degradation with each change in HAc concentrations at 2100, 3700, 4100, 6000, and 8200 mg HAc L^{-1} (Fig. 2). The highest concentrations of 6000 and 8200 mg HAc L^{-1} could be completely depleted in 5 d without observable lag phase (Fig. 2).

The lag phase details following HAc-shock loading and acclimation are as summarized in Table 2. While there was no observable lag phase during the first day of incubation, a zero-order linear relationship between HAc concentration and time was observed. The HAc degradation rate constants (k) and regression coefficients (R^2) are listed in Table 2. The HAc degradation rate after completion of acclimation at 8200 mg HAc L^{-1} was higher than those achieved following acclimation at 3700, 4100, and 6000 mg HAc L^{-1}, and all these HAc degradation rates were higher than the rate

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shown by unacclimated biomass at 4200 mg HAc L^{-1}.

3.2. Biomass activity during HAc-shock loading and acclimation

Adenosine triphosphate (ATP) can be regarded as an indicator of biomass activity (Lee et al., 2006), and it ubiquitously exists in all microorganisms (Hwang and Hansen, 1998). All living cells use ATP to conduct the essential energy-requiring reactions (Archibald et al., 2001). Hence, in this study, total ATP content was used to indicate biomass activity under different conditions. ATP content measured in this study included total ATP of all microbes.

When unacclimated biomass was incubated with 6400 and 7400 mg HAc L^{-1}, HAc degradation was delayed during the first day of incubation. Therefore, investigation of ATP content was focused on the first day of incubation, with comparisons at the 0^{th} d and the 1^{st} d for both acclimated and unacclimated biomass. As shown in Fig. 3, ATP content had increased from 0^{th} d to 1^{st} d while incubated with HAc concentrations wherein there was no observable lag phase (2100A, 3700A, 4100A, 6000A, 8200A, 2100S, and 4200S). This correlated with HAc degradation since it was an exergonic reaction coupled with ATP synthesis (Hattori, 2008). However, at the higher HAc concentrations of 6400 and 7400 mg HAc L^{-1} wherein there was a one day lag phase in the HAc degradation (6400S and 7400S), no increase in ATP content was noted during the same incubation period. Instead, a downtrend from the start of incubation was noted (Fig. 3). This could be related to the reduced biomass activity arising from stress from HAc accumulation during the first day of incubation (Fig. 1c and 1 d). ATP synthesis had been negatively affected by the HAc accumulation. The increase in ATP content in acclimated biomass at 8200 mg HAc L^{-1} compared to decrease in ATP content in unacclimated biomass at 7400 mg HAc L^{-1}

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during the first day of incubation suggested the acclimation strategy adopted could likely maintain biomass activity in face of increases in HAc concentration.

Since the inoculum used in this study was a mixed culture, therefore, the direct relationship between ATP and HAc inhibition could not be elucidated without excluding the impacts of other non-HAc utilizing microbes. However, with the evidence from this study, it could be suggested decrease in ATP content might be seen as an early-warning of HAc accumulation with negative impact on the biomass.

3.3. Microbial community profiles

Bacterial and archaeal communities in the acclimated and unacclimated second stage biomass were characterized with 454 pyrosequencing. Community richness (Chao 1, ACE, and number of observed OTUs) and diversity (Shannon index) were evaluated with the MOTHUR software (Supplementary data: Table S1 and S2). The present study had focused on HAc effects on the archaeal community. The rarefaction curves of these samples (Supplementary data: Fig. S2 and S3) approached asymptote phase, but did not reach a saturation status, indicating more archaeal phylotypes remained to be sampled. This maybe because environmental samples are usually abundant with rare species (Ashby et al., 2007). The Good's coverage of 96% - 98% showed a good sampling of the archaeal community (Lemos et al., 2011) (Table S1) was achieved. The unacclimated biomass at 7400 mg HAc L^{-1} (7400S) was found to have lower Chao1 (64.36 vs. 144.79), ACE (68.47 vs. 197.78), and Shannon's index (1.66 vs. 1.70) than at 2100 mg HAc L^{-1} (2100S) (Table S1). This indicated high HAc concentrations decreased the unacclimated biomass' microbial richness and diversity, and this could subsequently impair system stability. On the other hand, higher Chao1 (148.13 vs. 64.36), ACE (150.93 vs. 68.47), and Shannon's index (2.00 vs. 1.66) were

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observed in the acclimated biomass at 8200 mg HAc L^{-1} (8200A) compared to the unacclimated biomass at 7400 mg HAc L^{-1} (7400S). This indicated HAc acclimation restored microbial richness and diversity, and could alleviate the inhibition observed during shock-loading.

454 pyrosequencing analysis indicated the aceticlastic *Methanosarcinaceae* (MSC) and *Methanosaetaceae* (MST) were not present in the second stage, however the hydrogenotrophic *Methanomicrobiales* (MMB) represented 99.3% of the total archaeal population in the second stage, with minor presence of MBT (0.42%). The presence of *Methanolinea* (78.1%) and *Methanoculleus* (13.8%) (Supplementary data: Fig. S4), both belonging to the order MMB, were the dominant methanogens at genus level in the second stage. The carry-over of MMB from feed sludge (99.7%), seed sludge (99.8%), and the first stage (81.3%) (Supplementary data: Fig. S5) could be a reason for MMB predominance in the second stage. The stress conditions in the second stage, i.e. high salinity (8.79 ppt, ppt means parts per thousand), may be another reason for this predominance since the hydrogenotrophic methanogens (HM) were more competitive than aceticlastic methanogens at stress condition of high salinity (> 6 ppt) (Hao et al., 2010; Krakat et al., 2010).

The strain AOR, *Thermacetogenium.phaeum* (strain PB^T), *Thermotoga lettingae* (strain TMO), and *Clostridium ultunense* (strain BS^T) have been reported as syntrophic acetate-oxidizing bacteria (SAOB) present in anaerobic systems (Hattori, 2008). In this study, the first three types of microbes could not be identified at genus level. However, the genus *Clostridium* was 6.41% against total bacterial population in the second stage population. The preponderance of the hydrogenotrophic methanogenic (mainly *Methanomicrobiales*) and possible presence of acetate oxidizers (*Clostridium*) might suggest HAc-methanization was driven by the

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syntrophic acetate oxidation pathway in the second stage. However, further studies were needed to trace HM and SAOB roles in HAc degradation with the stable carbon isotopic signatures technique (Hao et al., 2011).

As shown in Fig. 1, biomass from the second stage showed tolerance up to 4200 mg HAc L^{-1}, which was higher compared to the typically reported values (2000 - 3000 mg HAc L^{-1}) (Mawson et al., 1991; Xiao et al., 2013). The predominance of HM (MMB) could have helped accommodate high HAc concentration, since HM has higher specific growth rate (Kim et al., 2013), less sensitivity, and better adaptation to toxic and shock conditions than aceticlastic methanogens (AM) (Krakat et al., 2010). Jang et al. (2014) report that HM dominated in the second-stage mesophilic bioreactor (HRT=19 d, pH uncontrolled) of a two-stage system for sludge treatment provide support. Hao et al. (2010) also reported HM dominated when treating sludge with high salt content (> 6 ppt).

For the acclimated and unacclimated biomass, MMB still remained dominant in proportion with 94.2% (2100S), 99.4% (4200S), 99.7% (7400S), 99.5% (3700A), and 99.6% (8200A) against the total archaeal community, respectively (Fig. 4a). The genus *Clostridium* was 3.43% (2100S), 4.68% (4200S), 4.99% (7400S), 2.29% (3700A), 2.30% (8200A) against the total bacterial community. Although the proportion of MMB had remained unchanged in both acclimated and unacclimated biomass, the 16S rRNA gene copy numbers of acclimated biomass were higher compared to the unacclimated biomass (Fig. 4b), with 2.43×10^{9} copies g^{-1} VSS (2100S) and 5.46×10^{9} copies g^{-1} VSS (7400S) in the unacclimated biomass increasing to 8.15×10^{9} DNA copies g^{-1} VSS in the biomass during acclimation (3700A) and 1.90×10^{10} DNA copies g^{-1} VSS in the biomass after completion of acclimation (8200A). This increase in the archaeal population would be related with the higher HAc

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degradation rates of the acclimated biomass at 3700 and 8200 mg HAc L^{-1} compared to the unacclimated biomass at 2100 and 7400 mg HAc L^{-1} (Table 2) since more energy would have been generated for biomass (i.e. archaea) growth with the higher HAc utilization (Grady Jr et al., 2011). Although the HAc degradation rate after completion of acclimation at 8200 mg HAc L^{-1} was higher than those achieved following acclimation at 3700 mg HAc L^{-1} (Table 2), the specific HAc degradation rate may be not necessarily increasing since the 16S rRNA gene copy numbers were increased.

The stability of the two-stage anaerobic system is a current topic for investigation in recent years since its superiority was often compromised by decreased methanogen activity due to VFAs accumulation in the second stage (Schievano et al., 2012). The tolerance of second stage biomass to HAc inhibition would be important for overall stability of the two-stage anaerobic system. The stepwise acclimation process could enrich an appropriate number of specific microbes, with increased tolerance to the inhibition. For example, Lins et al. (2012) improved start-up performance with stepwise acclimation to overcome high HAc inhibition (9000 mg HAc L^{-1}). Chen et al. (2003) had increased the sodium concentration causing 100% inhibition of thermophilic methanogen activities from 10.6 g Na^+ L^{-1} to 22.8 g Na^+ L^{-1} with stepwise sodium acclimation of 4.1, 7.1 and 12.1 g Na^+ L^{-1}. The use of stepwise acclimation in this study had also succeeded in accommodation of substantially higher levels of HAc accumulation. This stepwise acclimation approach might be applied in full-scale two-stage anaerobic plants by controlling the operational conditions, such that HAc production in the first stage is increased “stepwise” so as to achieve stepwise HAc concentration increase in the feed to the second stage. This may be achieved by manipulating the loading rates and thereby providing opportunity for

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375 enriching MMB.

376

377 **4. Conclusions**

378 In this study, biomass from the second stage of a two-stage anaerobic system
379 could degrade HAc up to 4200 mg HAc L^{-1} without observable lag phase. The
380 preponderance of *Methanomicrobiales* (99.3%) and presence of *Clostridium* (6.41%)
381 were noted in the population in the second stage. The proportion of
382 *Methanomicrobiales* remained dominant (95 %) as methanogenic populations grew
383 during HAc stepwise acclimation, and possibly allowed for higher HAc degradation
384 ability to 8200 mg HAc L^{-1}. The results suggested the stepwise acclimation could be
385 an approach to accommodate HAc accumulation and hence higher concentrations.
386 The key to higher HAc tolerance within the second stage of a two-stage system would
387 be to develop conditions which allowed higher abundance of *Methanomicrobiales*
388 therein as the first stage became more effective at hydrolysis and acidogenesis.

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392

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