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# Effects of free ammonia on volatile fatty acid accumulation and process performance in the anaerobic digestion of two typical bio-wastes

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ABSTRACT

The effect of free ammonia on volatile fatty acid (VFA) accumulation and process instability was studied using a lab-scale anaerobic digester fed by two typical bio-wastes: fruit and vegetable waste (FVW) and food waste (FW) at 35°C with an organic loading rate (OLR) of 3.0 kg VS/($m^3$·day). The inhibitory effects of free ammonia on methanogenesis were observed due to the low C/N ratio of each substrate (15.6 and 17.2, respectively). A high concentration of free ammonia inhibited methanogenesis resulting in the accumulation of VFAs and a low methane yield. In the inhibited state, acetate accumulated more quickly than propionate and was the main type of accumulated VFA. The co-accumulation of ammonia and VFAs led to an "inhibited steady state" and the ammonia was the main inhibitory substance that triggered the process perturbation. By statistical significance test and VFA fluctuation ratio analysis, the free ammonia inhibition threshold was identified as 45 mg/L. Moreover, propionate, iso-butyrate and valerate were determined to be the three most sensitive VFA parameters that were subject to ammonia inhibition.



## Introduction

The rapid increasing disposal of municipal solid wastes (MSWs) has resulted in severe environmental problems in China. Two types of typical bio-wastes, including fruit and vegetable waste (FVW) and food waste (FW), contributed to high organic and water content in MSW (Zhang et al., 2014). As compared with conventional treatment technologies, anaerobic digestion has emerged as one of the most promising alternative technologies for the treatment of high organic content waste as well as recovery of renewable energy-biogas (De Clercq et al., 2016). Despite this finding, various operational problems still prevent the anaerobic process from being widely applied. The substrates' compositions, usually referred as C/N ratio and ammonia concentration, are considered to be the key parameters affecting process stability and performance (Mata-Alvarez et al., 2014; Yuan and Zhu, 2016).

An optimum C/N ratio in the range of 20 to 30 is essential for anaerobic digestion which can help to keep an appropriate nutrient balance for the microbial growth and to maintain a stable environment (Li et al., 2015; Mata-Alvarez et al., 2014). Therefore, the anaerobic digestion performance of low C/N ratio substrate, such as FW and FVW, is usually not very effective and stable. Low C/N ratio substrates contain a relatively higher percentage of nitrogenous organic matters. Ammonia produced by the biological degradation of nitrogenous organic matters

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was in excess for microorganism utilizing. The excess ammonia usually accumulates during the process and leads to an increase of pH, inhibitory effects, and eventually, process deterioration (Ariunbaatar et al., 2015; Sheng et al., 2013). Ammonium ion ($NH_4^+$) and free ammonia (FA) ($NH_3$) are the two principal forms of total ammonia nitrogen (TAN) of which FA has been suggested to be the main cause of inhibition. Hydrophobic free ammonia molecules may diffuse passively through the membrane and into the cell, resulting in proton imbalance and/or potassium deficiency (Belmonte et al., 2011; Chen et al., 2008). A high concentration of ammonia can inhibit methanogenesis resulting in the accumulation of volatile fatty acids (VFAs) and, as a result, low methane yield. In the literature, the inhibitory concentrations of TAN and free ammonia were in the range of 1500–7000 mg/L and 53–1450 mg/L, respectively (Rajagopal et al., 2013). This wide range is mainly due to the different substrates, inocula, environmental conditions (affecting pH and temperature, for instance) and acclimation (Chen et al., 2008; Rajagopal et al., 2013; Yenigün and Demirel, 2013).

Previous research on ammonia inhibition in anaerobic digestion has mainly been focused on inhibition concentration thresholds, inhibition mechanisms, and microbial community shift, *etc.* (Gao et al., 2015; Poirier et al., 2016; Rajagopal et al., 2013). Furthermore, feasible and sensitive indicators of the anaerobic digestion (AD) process subject to ammonia inhibition are equally important to monitor system health and to prevent the system from collapse. Parameters of biogas production rate, methane yield, pH, VFAs, *etc.*, have been recommended as the process indicators; however, these parameters are either not sensitive enough to reflect the process instability or not feasible for *in situ* measurement (Nielsen et al., 2007). Biogas production rate and methane yield are the most commonly used monitoring indicators, but these indicators respond slowly and as a result, cannot indicate process instability timely (Boe et al., 2010; Nielsen et al., 2007). The pH measurement is easy to obtain, but not reliable when used in highly buffered systems. Under such conditions, even rapid increases in VFAs or ammonia cannot lead to significant pH fluctuation. Many researchers have suggested that VFAs could be good indicators of the process. VFAs are the most predominant intermediates during the AD process and their accumulation indicates the imbalance between sequential steps of AD process (Boe et al., 2010; Madsen et al., 2011). Ahring et al. (1995) suggested that butyrate and iso-butyrate concentration might be reliable for indicating process instability because they are sensitive to different types of perturbation imbalances. Nielsen et al. (2007) suggested that propionate might be the best indicator during a process disturbance caused by overloading because it has proven significant and long-lasting. Nakakubo et al. (2008) studied thermophilic digestion of pig manure with intermittent $NH_4Cl$ pulsing, and found that iso-butyrate, butyrate, and iso-valerate, rather than propionate, were useful indicators for acute ammonia induced perturbation. Two of the most abundantly produced VFAs (*i.e.*, acetate and propionate) did not accumulate with increased ammonia concentration.

In addition to being intermediates and indicators, VFAs are also essential buffering agents in the AD system. Moreover, high concentrations of VFAs show an inhibitory effect to methanogenesis (Yuan and Zhu, 2016). VFAs accumulate at a high organic loading rate or during perturbations when methanogens cannot utilize hydrogen and VFAs as quickly as they are produced by acidogens and acetogens. High concentrations of free VFAs are thought to freely permeate the cellular membrane and damage the macromolecules in low-pH environments, especially for the gram-positive bacteria (Wang et al., 2009; Yuan and Zhu, 2016). Accumulation of VFAs leads to rapid pH decrease, and eventually, process deterioration. Wang et al. (2009) reported that a propionate concentration of 900 mg/L resulted in the significant inhibition of methanogens. Xu et al. (2014) found that acetic acid was the main VFA inhibitor in methanogenesis when treating kitchen wastes. The initial inhibitory concentration of acetic acid was between 1.5 and 2.5 g/L and the methanogenesis activities were inhibited completely at the VFA concentration of 5.8–6.9 g/L.

Ammonia and VFAs are both inhibitory to methanogenesis and can lead to pH fluctuation. High concentrations of both ammonia and VFAs usually lead the system to fall in an "inhibited steady state", in which the digester runs stably within a neutral pH range, but where methane production rate and volatile solid (VS) reduction rates are quite low (Angelidaki and Ahring, 1993; Chen et al., 2008). Although the individual effects of VFAs or ammonia on methanogenesis have been widely reported, comprehensive analysis of ammonia–VFA interaction has seldom been demonstrated. Whether ammonia or VFAs are the main inhibitory substances that trigger the process perturbation is undefined. Finding useful indicators for potential process perturbation is also unclear. Thus, in this study, the anaerobic digestion of two kinds of typical bio-wastes was conducted to investigate the ammonia inhibition effects on VFA accumulation and process performance. The interaction of ammonia-VFAs, which led the digester into an "inhibited steady state" was also studied. In addition, this study aimed to identify an ammonia inhibition threshold and to determine sensitive VFA parameters as indicators subject to ammonia inhibition. This study provided useful insight into preventing ammonia inhibition from causing low efficient biogas production and process deterioration.

## 1. Materials and methods

### 1.1. Substrates and inocula

Raw FVWs were collected from a fruit and vegetable market in Beijing in during July to January of the next year. The FVW mainly contained residues of Chinese cabbage, carrot, lettuce, apple, banana, and watermelon. Raw FW, which mainly consists of leftovers from cooked foods, was collected from students' restaurants in Tsinghua University, Beijing, China. The FWV and FW were pre-treated and homogenized using a food grinder after manually sorting out bones, paper and plastics *etc.* The samples were stored at 4°C before use. Inocula were anaerobic granular sludge taken from a full-scale upflow anaerobic sludge bed (UASB) reactor treating starch processing wastewater at 35°C. The granular sludge was ground into slurry before the experiment. The characteristics of substrates and inocula are summarized in Table 1 (Lin et al., 2011).

**Table 1 – Characteristics of the fruit and vegetable wastes, food wastes and inocula (Lin et al., 2011).**

| | pH | Total solid (%) | Volatile solid (%) | Elemental composition (wt.% TS) | | | | BMP ($Nm^3$ $CH_4$/kg VS) |
|---|---|---|---|---|---|---|---|---|
| | | | | C | H | O | N | |
| FVW | 4.69 ± 0.89 | 7.9 ± 1.4 | 6.7 ± 1.0 | 43.3 | 5.2 | 38.0 | 2.8 | 0.30 ± 0.02 |
| FW | 4.05 ± 0.50 | 21.3 ± 1.2 | 19.3 ± 1.1 | 51.0 | 7.3 | 29.2 | 3.0 | 0.56 ± 0.03 |
| Inocula | 7.21 ± 0.04 | 12.5 ± 0.5 | 9.9 ± 0.3 | ND | ND | ND | ND | ND |

BMP: biomethane potential; VS: volatile solid; FVW: fruit and vegetable wastes; FW: food wastes; ND: not determined.

### 1.2. Operational conditions of semi-continuous reactor

A lab-scale continuous stirred tank reactor (CSTR) was operated for 178 days. The reactor was made of polymethyl methacrylate with a height of 380 mm and inner diameter of 140 mm (Appendix A Fig. S1). The total volume of the reactor was 6 L and the working volume was 4 L. A thermostat water jacket was applied to keep the temperature at 35 ± 1°C. A mechanical stirrer (120 r/min) was used for intermittent mixing in digester with 20 min on every hour throughout the experiment. The substrates were fed into and effluents were drawn out of the digester once per day at a consistent organic loading rate (OLR) of 3 kg VS/($m^3$·day). The experiment was divided into 5 phases with different FVW and FW substrate proportion. The experiment began with only FVW as a substrate in Phase I, and then FW proportion (based on volatile solid contents) increased to 33%, 50%, 67%, and 100% in Phases II–V stepwise. Although the C/N ratio of FVW and FW was similar, their biodegradability was different. The FW were easier to hydrolyze and degrade compared with the FVW and released ammonia more quickly. Accordingly, gradual ammonia accumulation was realized at consistent organic loading rate without addition of external ammonia nitrogen.

### 1.3. Batch experiment

The batch tests were conducted at 35 ± 1°C, using the OxiTop® Control AN 6 BOD measurement for biogas determination systems (WTW GmbH, Germany), with 6 measuring points, one controller and one stirring platform. Bottles had a liquid volume of 300 mL and headspace volume of 700 mL (Appendix A Fig. S2). The granule sludge was rinsed and adjusted to 1.2 g VS/L with tap water. 10 g glucose was used as the single substrate, and $NH_4Cl$ was added to control the TAN concentration to 0, 500, 1000, 2000, and 4000 respectively. A blank control was set without $NH_4Cl$ or the addition of glucose. The pH value was adjusted to 7.8 at the beginning of the test. The batch tests were conducted for 24 hr, and the concentration of VFAs was measured afterward.

### 1.4. Analytical methods

TS (total solid) and VS (volatile solid) of the samples were determined directly according to APHA Standard Methods (APHA, 2005). The pH values of samples were determined by a pH meter. All of the samples were analyzed in triplicates. Samples were centrifuged at 15,000 r/min for 20 min, and then the supernatant filtered through a 0.45-μm cellulose acetate membrane. Afterward, sCOD, N-$NH_4^+$ were measured according to APHA Standard Methods (APHA, 2005). VFAs concentrations were determined using a gas chromatography (6890 N, Agilent, USA) equipped with a flame ionization detector and a capillary column (25 m × 0.32 mm × 0.5 μm; Hewlett Packard-FFAP, USA). The volume of the biogas was measured using a flow meter (Duo yuan LML-2, China). The elemental compositions of raw substrates were analyzed by an elemental analyzer (CE-440, EAI Co., USA). Biomethane potential (BMP) was measured following a standard protocol (Angelidaki et al., 2009).

### 1.5. Calculations

Assuming that organic matters in FVW and FW can be represented with the formulation of $C_aH_bO_cN_d$ and that all organic components are converted into $CH_4$ and $CO_2$, the theoretical methane potential (TMP) was estimated using the Eqs. (1) and (2) (Sosnowski et al., 2003):

$$C_aH_bO_cN_d + \frac{(4a-b-2c+3d)}{4}H_2O \rightarrow \frac{(4a+b-2c-3d)}{8}CH_4 + \frac{(4a-b+2c+3d)}{8}CO_2 + dNH_3 \quad (1)$$

$$V_T = \frac{(4a+b-2c-3d)\times 2.8}{12a+b+16c+14d} \quad (2)$$

The biodegradability was calculated using Eq. (3), in which $V_T$ was the TMP and $V_C$ was the cumulative methane production obtained from the BMP test (Penaud et al., 1999).

$$\text{Biodegradability} = \frac{V_C}{V_T} \quad (3)$$

The free ammonia (FA) concentration was calculated according to Eq. (4) (Belmonte et al., 2011):

$$C_{FA} = \frac{C_{TAN}}{1+\frac{10^{-pH}}{K_a}} \quad (4)$$

where, $C_{FA}$ and $C_{TAN}$ are the free ammonia and the total ammonia nitrogen concentration, respectively. $K_a$ is the dissociation constant, with values $1.097 \times 10^{-9}$ at 35°C.

In the semi-continuous experiment, the VFA concentration remains relatively constant during days 1–94, which was set as the base period for monitoring VFA concentration. An abrupt VFA accumulation was measured during days 95–131 and could be determined as VFA response subject to ammonia inhibition. To evaluate individual VFA concentrations responses subject to

ammonia inhibition, a significant value (z) is calculated as (Ahring et al., 1995):

$$z = \frac{C - C_0}{SD} \quad (5)$$

where, $C$ (mg/L) is the measured value of the VFA concentration, $C_0$ (mg/L) is the average value of the VFA concentration of the base period, and SD is the standard deviation of the VFA concentration of the base period.

To evaluate the individual VFA concentration's relative fluctuation, the fluctuation ratio of VFA concentration ($r$) is calculated as (Nakakubo et al., 2008):

$$r = \frac{C - C_0}{C_0} \quad (6)$$

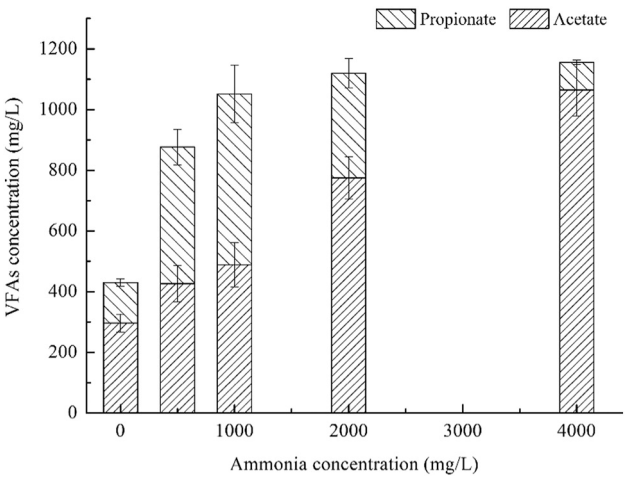


**Fig. 1 – Volatile fatty acid concentrations at different ammonia concentrations in batch tests.**

## 2. Results and discussion

### 2.1. C/N ratios and biodegradability of FVW and FW

According to the elemental compositions of FVW and FW in Table 1, the C/N ratio of FVW and FW was 15.6 and 17.2 respectively, which was lower than the numbers suggested in the literature for the stable operation of the digester. This indicated that FVW and FW contained a relatively large quantity of nitrogen, mainly in protein forms. Accordingly, the digestion of FVW and FW may not be successful due to the potential risk of ammonia inhibition.

The organic composition of FVW and FW was represented as $C_{18.2}H_{26.2}O_{12}N$ and $C_{20.1}H_{34.7}O_{8.6}N$ based on elemental composition and Eq. (1). Therefore, the theoretical methane potential (VT) of FVW and FW was 0.51 and 0.67 $Nm^3$ $CH_4$/kg VS, respectively. Based on TMP and BMP values, the biodegradability of FVW and FW was determined as 59.3% and 83.6%, respectively. FVW had a lower biodegradability as compared to FW because it contained a higher composition percentage of cellulose and hemicellulose. Moreover, FW contained generally 53% carbohydrates, 16% protein and 22% fat, *etc.*, which were easier to be hydrolyzed and degraded (Zhang et al., 2014). Although both FVW and FW contain a high composition of organic nitrogen, the organic matter in FW was degraded more quickly and ammonia would accumulate quicker in the digester.

### 2.2. Ammonia inhibition effects on VFA accumulation

The results of the batch tests showed that after one day's consumption of glucose, the five batch reactors with different initial added ammonia concentration levels had different acetate and propionate concentration (Fig. 1). The acetate concentration increased from 296 to 1064 mg/L with increasing ammonia concentration. The propionate concentration increased from 133 to 562 mg/L when the initial added ammonia concentration increased from 0 to 1000 mg/L, while the propionate decreased from 562 mg/L to 91 mg/L when the initial added ammonia concentration increased from 1000 to 4000 mg/L. Moreover, the P/A (propionate/acetate) ratio increased at first and then decreased with the increase of the initial ammonia concentration. When the initial added ammonia was 1000 to 4000 mg/L, the total concentration of acetate and propionate was similar but the proportion of acetate increased proportionally with the increasing ammonia concentration. High ammonia concentration caused more severe acetate accumulation than propionate.

The accumulation of a certain VFA was due to unbalanced VFA production and utilization. Acetate was consumed by aceticlastic methanogens to produce methane while acetate was produced by the degradation of propionate, butyrate, *etc.* Propionate was consumed by propionate degradation to produce acetate and $H_2$ while propionate was produced by degradation of valerate, *etc.* Propionate degradation was considered to be the most thermodynamically unfavorable step in the AD system. Propionate degradation was only thermodynamically favorable under relatively low hydrogen partial pressure. High hydrogen partial pressure would inhibit the propionate degradation and also indicate that the hydrogenotrophic methanogens were inhibited (Ketheesan and Stuckey, 2015). In our test, the severer acetate accumulation indicated that under ammonia inhibitory conditions, acetate-consuming methanogens were inhibited. Furthermore, moderate propionate accumulation indicated that the propionate degradation was not as inhibited as acetate degradation. The acetate was the main type of accumulated VFA (rather than propionate) under ammonia inhibition which was different from other perturbations, such as overloading and acidification. These results could be explained by different ammonia tolerance of aceticlastic methanogens and hydrogenotrophic ones. Numerous studies have found that ammonia had strong inhibitory effects for the methanogens and that aceticlastic methanogens were less tolerant of ammonia than hydrogenotrophic ones (Lü et al., 2013; Fotidis et al., 2013; Westerholm et al., 2012). Under a high ammonia concentration, the aceticlastic methanogens were heavily inhibited and acetate accumulated simultaneously. Under a low ammonia concentration, the hydrogenotrophic

methanogens were just slightly inhibited and the hydrogen partial pressure remained low. Low hydrogen partial pressure kept the propionate degradation thermodynamic favorable so that propionate would not substantially accumulate.

### 2.3. Ammonia, VFA concentration and pH in different operational phases

The semi-continuous reactor was operated for 178 days in 5 phases. During the 5 phases, the organic loading was consistent, at 3 kg VS/($m^3$·day), and the FW proportion (based on VS) in the substrates increased from 0%, to 33%, 50%, 67%, 100%, stepwise. Increasing the influent proportion of FW led to gradual ammonia accumulation without the addition of external ammonia nitrogen because that organic ammonia nitrogen in FW was easier to degrade and release, compared with FVW.

Generally, the anaerobic digester was a buffered system and the pH value depended on the relative concentration of buffering agents in the anaerobic system. Weak acid and alkaline substances such as VFAs, carbonates, bicarbonates, ammonia and sometimes sulfides are principle forms of buffering agents. Because the concentration changes of (bi-) carbonates is usually small, the pH value fluctuation is mainly due to ammonia and/or VFA accumulation. As shown in Fig. 2a, in the first four phases, despite the low pH of the substrate (4.24–5.14), the pH value in the digester remained consistent within a relatively stable neutral range of 7.20 to 7.85. Because VFAs did not accumulate much, the rising ammonia concentration to a gradual increase in pH value (from 7.20 to 7.85). In Phase V, when the FW was a single substrate, ammonia inhibition caused VFA accumulation. A high concentration VFAs would deplete the buffering capacity in the digester and cause a pH decrease from 7.84 (highest) to 6.78 (lowest) in 36 days. At the end of the experiment, the pH value of the digester was relatively stable and in neutral range, with a high concentration of both ammonia and VFA.

Ammonia was produced by the biological degradation of nitrogenous organic matters in substrates, which mainly were proteins, phospholipids, nitrogenous lipids, *etc.* The organic

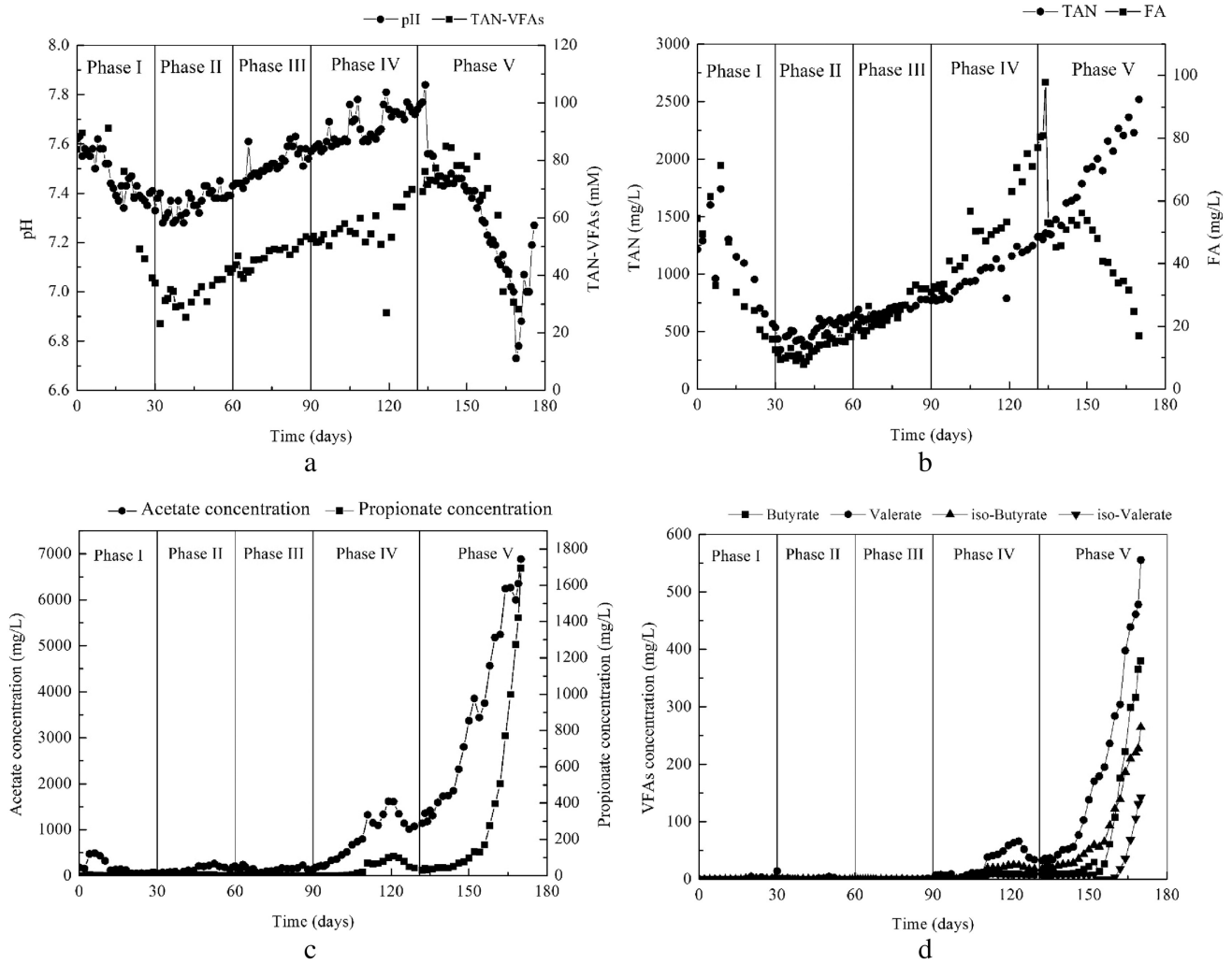


**Fig. 2 – pH, ammonia and VFA concentration in different operation phases.(TAN: Total ammonia nitrogen, FA: Free ammonia). a: TAN and FA; b: pH and TAN-VFA molar concentration; c: acetate and propionate concentration; d: (iso-) Butyrate, (iso-) Valerate concentration.**

nitrogen in the FW was more quickly hydrolyzed I into soluble ammonia nitrogen compared to FVW. As shown in Fig. 2b, in Phase I, the ammonia concentration first decreased from 1215 to 535 mg/L. This was because of the poor degradability of the FVW and the slow release of ammonia. In Phases II–V, the ammonia concentration increased from 435 to 2518 mg/L with an increasing influent FW proportion. According to Eq. (4), an increase in pH would result in a higher FA to ionized ammonia ($NH_4^+$) ratio; thus, the FA concentration increased from 9 (lowest value) to 97 mg/L (highest value) which was more significant than ammonia. The FA was suggested as the main cause for methanogen inhibition. In the last 10 days of Phase IV (days 121–131), even the TAN concentration was below 1500 mg/L and much lower than the inhibition threshold concentration reported in the literatures. The FA concentration was between 63 and 77 mg/L, which was already in the range of inhibition concentration (Massé et al., 2003; Rodriguez et al., 2011; Sung and Liu, 2003).

Among the four types of anaerobic microorganisms, the methanogens are suggested as the least tolerant and most likely to cease growth due to ammonia inhibition (Chen et al., 2008). The inhibitory effects of free ammonia were reflected by the increasing concentration of VFAs. As shown in Fig. 2c and d, in the first three phases, the VFAs maintained a lower concentration level as well as lower ammonia concentration and stable pH value, which indicated that the system was stable and the VFAs were well consumed by syntrophic acetogens and methanogens. In Phase IV, the FW influent proportion was at 67% and the rising ammonia concentration caused inhibitory effects on methanogenesis, while the other three types of anaerobic microorganisms remained unaffected. A higher proportion of easy biodegradable FW also led to a higher VFA production rate. This caused an imbalance in VFA production and utilization and a rapid accumulation of VFAs, from 1200 to 9900 mg/L. The ammonia inhibition resulted in the accumulation of VFAs, especially acetate. Acetate could be cleaved into methane and carbon dioxide (bicarbonate) by aceticlastic methanogens. A significant rising concentration of acetate indicated that the aceticlastic methanogens were inhibited by ammonia. Propionate was also suggested to be a key parameter indicating system instability because propionate degraders were often the slowest growing and energetically most sensitive microorganisms in the AD process (Nielsen et al., 2007). In Phase IV, the propionate concentration increased along with a higher influent FW proportion, but not very significantly and generally at a rate lower than 100 mg/L. A relatively lower propionate concentration indicated that the propionate degradation was slightly inhibited even with the product inhibition of acetate. The results were in accordance with our batch tests in which the acetate was the main type of accumulated VFA (rather than propionate) under ammonia inhibition. In Phase V, a high concentration of VFAs (especially propionate) was also inhibitory to methanogens. The decreasing pH lowered the FA concentration and ammonia inhibition was alleviated to some extent, but the VFAs became new main inhibitors. In this process, the proportion of acetate to total VFAs deceased from approximately 90% to 70%. This was mainly due to the inhibitory effects of acetate production on propionate degradation.

The results also showed interactions between ammonia, VFA and pH. The fluctuation of pH was almost simultaneous with the fluctuation of TAN-VFA molar concentration. When both TAN and VFAs remained at low levels, the pH remained at 7.3 to 7.5; however, when the ammonia first accumulated, the pH increased to approximately 7.8, the VFA accumulated shortly after the ammonia accumulation. When both TAN and VFAs accumulated to a high level, the pH finally remained at 7.2. Under such conditions, although the pH was neutral and relatively stable, the high concentration of ammonia and VFAs inhibited methanogenesis and the biogas production rate was approximately 85% lower than the rate before the disturbance. In this period, the system was in an "inhibited steady state" with a stable pH value and low biogas production rate. The results also showed that the ammonia was the main inhibitory substance triggering the process perturbation, which eventually fell into an "inhibited steady state."

### 2.4. Biogas production rate in different operational phases

Biogas production rate (BPR) is one of the most important indicators in the AD process because it reflects the methanogen activity and stability of the system. As shown in Fig. 3, in the first four phases, the biogas production rate was relatively stable, from 2.17 to 2.45 $m^3/(m^3 \cdot day)$, and slightly increased with the influent FW proportion. When ammonia inhibition happened in Phase IV, the BPR was just slightly affected. This may be due to a methanogensis pathway shift. It has been reported in the literature that hydrogenotrophic methanogens were more tolerant of ammonia inhibition than aceticlastic methanogens. When aceticlastic methanogens were inhibited, there would be a microorganism shift from aceticlastic methanogensis to hydrogenotrophic methanogensis (Fotidis et al., 2013; Schnürer and Nordberg, 2008; Westerholm et al., 2012). The results also showed that the BPR was maximized in Phase IV with an influent FW proportion of 67%; however, under such operating conditions, the system was not stable. In Phase V, the BPR declined rapidly when the FW was the single substrate. This was because the methanogens have not recovered from the ammonia inhibition and rapid

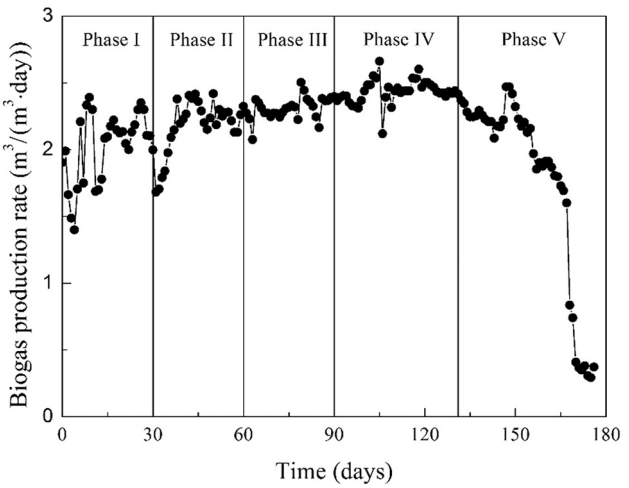


**Fig. 3 – Biogas production rate (GPR) in different operation phases.**

accumulation of VFAs also led to VFA inhibition which caused severe inhibition of microbial, especially the methanogens. As we discussed above, the methanogensis was severely inhibited in Phase V, while the hydrolysis and acidogenesis was only slighted affected because the ammonia and VFA concentration were consistently increasing. Even when the pH value finally remained stable and neutral at 7.2, the BPR did not recover from the VFA and ammonia inhibition and, therefore, was approximately 85% lower. The results of BPR demonstrated that it was a valuable indicator, but not sensitive. The BPR fluctuation was much later than the time when the system began to deteriorate. Additionally, BPR was influenced by the organic loading rate and the substrates' characteristics.

### 2.5. VFAs as indicators of system instability

To find the most sensitive individual VFA parameters as the system indicator, a serial of VFA significance test was conducted (shown in Table 2). Because the ammonia inhibition was observed in Phase IV, we mainly focused on days 91 to 131 (Phase IV) when the VFAs began to accumulate due to ammonia inhibition. The results of the significance test (Table 2) indicated that VFAs began to accumulate significantly on days 101–107. During this period, on day 99, acetate was the first VFA to accumulate, while propionate, butyrate, iso-butyrate, valerate, iso-valerate began to accumulate on days 105 and 107. The results indicated that a free ammonia concentration of approximately 45 mg/L (average value of days 99–107) may be the threshold of ammonia inhibition. Of all six types of VFAs, propionate, iso-butyrate and valerate were the three most sensitive parameters. Two combined parameters, *i.e.*, Bu + iBu (Butyrate + iso-Butyrate) and Va + iVa (Valerate + iso-valerate), were also calculated (not shown here). The two parameters were also among the most sensitive parameters. As for acetate, it accumulated to a relatively high absolute concentration due to ammonia inhibition. However, the relative acetate concentration was not as sensitive as other types of VFAs at the beginning of ammonia inhibition. This result was similar with Ahring's study that a combination of butyrate and iso-butyrate was a reliable indicator of system instability (Ahring et al., 1995). Nielsen et al. (2007) also suggested that propionate was a key parameter which was sensitive and persistent during the anaerobic digestion process.

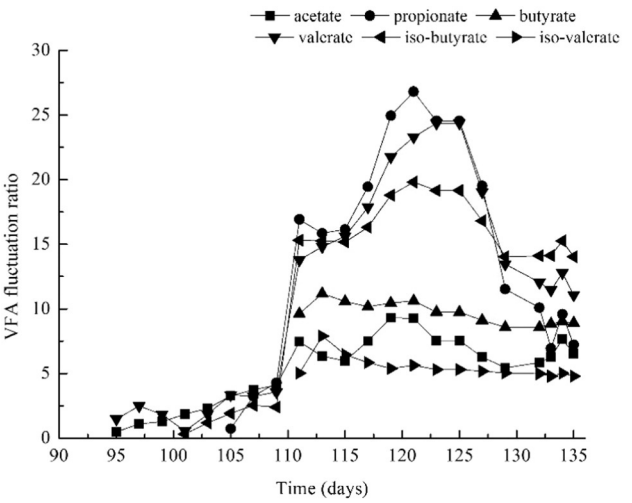


**Fig. 4 – VFA fluctuation ratio in days 95–135.**

The fluctuation ratio of VFAs concentrations was also calculated and shown in Fig. 4. The base period level was set as the average concentration of the period from days 1 to 94. A significant increase in all types of VFAs concentration was observed on day 111. Similarly, propionate, iso-butyrate and valerate were the three most sensitive parameters of the six VFAs. Bu + iBu and Va + iVa were also sensitive parameters subject to ammonia inhibition. These results were in accordance with the significance test.

## 3. Conclusions

The inhibitory effects of ammonia on methanogenesis were observed due to a low C/N ratio of FW and FVW (15.6 and 17.2 respectively). In the batch experiment, acetate was found to accumulate quickly, while propionate accumulated relatively slowly. This phenomenon demonstrated that the acetate was the main type of accumulated VFA rather than propionate under ammonia inhibition – which was different from other perturbations, such as overloading or acidification. In the semi-continuous experiment, the increasing FW proportion in

**Table 2 – Significance test of VFAs from day 95–111.**

| Day | Acetate | Propionate | Butyrate | Valerate | Iso-butyrate | Iso-valerate |
|---|---|---|---|---|---|---|
| 95 | 0.82 | ND | ND | 1.57 | ND | ND |
| 97 | 1.84 | ND | ND | 2.62* | ND | ND |
| 99 | 2.13* | ND | ND | 1.94 | ND | ND |
| 101 | 3.11* | ND | ND | 0.60 | 0.40 | ND |
| 103 | 3.79* | ND | ND | 1.95 | 1.53 | ND |
| 105 | 5.42* | 1.23 | ND | 3.53* | 2.44* | ND |
| 107 | 6.20* | 5.40* | ND | 3.41* | 3.24* | ND |
| 109 | 6.78* | 7.10* | ND | 3.77* | 3.07* | ND |
| 111 | 12.3* | 28.1* | 15.6* | 14.6* | 19.6* | 7.58* |

ND: not determined.
* Significant at 5% level.

influent during 5 phases led to a gradual accumulation of ammonia. Moreover, the high ammonia concentration resulted in the accumulation of VFAs. In the inhibited state, acetate accumulated quickly, and accounted for approximately 90% of total VFAs. The co-accumulation of ammonia and VFAs resulted in a stable and neutral pH value, but a low BPR known as an "inhibited steady state". The ammonia was proven to be the main inhibitory substance that triggered the process perturbation. The biogas production rate was a valuable indicator, but not sensitive and reliable. VFAs were one of the most important intermediate products and inhibitors in the anaerobic digestion process and they were confirmed to be very good indicators of the process. By significance testing and VFA fluctuation ratio analysis, the free ammonia inhibition threshold was identified to be 45 mg/L. Meanwhile, propionate, iso-butyrate and valerate were identified as the three most sensitive VFA parameters subject to ammonia inhibition.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2016.07.006.

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