



# Underestimated humic acids release and influence on anaerobic digestion during sludge thermal hydrolysis

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## ABSTRACT

Humic-like acids (HAs) are abundant in sewage sludge but mainly bonded with solids. Thus, their influences are often neglected in conventional digestion processes. Currently thermal hydrolysis pretreatment (THP) has been widely adopted in sludge anaerobic digestion (AD) to enhance hydrolysis of complex matters and further to improve methane production. However, the impacts of enhanced release of HAs and the mechanisms involved are not well understood and need to be further investigated because the substantial amounts of HAs present in AD could severely threaten the sludge AD processes. Results in the present study indicated that the concentration of soluble HAs in sludge was elevated by 90 times due to the THP, from 8 mg/L in raw sludge to 727 mg/L in the pretreated sludge hydrolyzed at 180 °C. Moreover, the structural characteristics of soluble HAs, including aromatic condensation degree, elemental composition and functional group, also showed substantial differences with the increased temperature of the THP. Furthermore, the release of HAs presented significant influences on sludge digestion. Acidification rate was inhibited by over 50% with 0.4 g/L of HAs, whereas methanogenesis was improved by nearly 200% with 0.8 g/L HAs and inhibited about 50% with 2.0 g/L. The activities of proteinase and co-enzyme F<sub>420</sub> were decreased by 20% and increased by 19%, respectively, under HAs stress at 0.6 g/L for 5 days. Moreover, molecular structural changes of soluble HAs also contributed to the influences. Especially, the E4/E6 value representing the degree of HAs aromatic condensation and C/N ratio of soluble HAs were closely correlated with their inhibition degree to sludge hydrolysis. The findings of this study demonstrate that the influences of HAs are evident and also vary to the different steps of anaerobic digestion processes, which shall not be negligible during the sludge digestion that is with THP. Due to the rate-limiting step was methanogenesis in the AD process of pretreated sludge by thermal hydrolysis, HAs concentration was recommended at low level, for example around 1.0 g/L, to accelerate or not limit methanogenesis.

## 1. Introduction

Approximately 12 million tons dry municipal sludge each year are produced in China, and sludge disposal takes almost 40–60% of the total operational cost in (wastewater treatment plants) WWTPs (Liu et al., 2017). Methane production from the sludge is a promising technology, which can not only effectively reduce the environmental hazards of waste activated sludge (WAS), but also generate clean energy (Liu et al., 2018; Mao et al., 2015; Vasco et al., 2018). However, relatively low conversion rates of organics limit the rates of methane production and reduction of sludge in terms of chemical oxygen demand (COD) or volatile solid (VS) related to volume reduction, dewaterability and

stability, which subsequently restricts the overall economic benefits (Curves et al., 2009; Liu et al., 2016; Yuan et al., 2019).

Nowadays, thermal hydrolysis has been widely employed to enhance the performance of AD. During sludge hydrolysis, cell walls of microbes break down, particulate organic matter and cellular materials can be disintegrated, and soluble microbial by-products (SMPs) and extracellular polymeric substances (EPSs) are hydrolyzed into small molecular organics (Gonzalez et al., 2018; Choi et al., 2018; Hii et al., 2019). However, some inhibitory substances, such as HAs, are also released into the supernatant, resulting in an increase of residual organic matter. Previous results showed that although the total content of HAs in sludge remained constant during thermal treatment, over 35% of humic and

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fulvic acids were solubilized into the liquid from the solids. The concentration of soluble humic substances could be increased from 0.1 to 1.0 g/L to 1.0–8.0 g/L, and even up to 8.0–15.0 g/L in high-solid sludge AD (Ghasimi et al., 2016; Liu et al., 2019; Tang et al., 2018)

With the wide applications of thermal hydrolysis in sludge pretreatment, the impacts of HAs on sludge AD has attracted more and more attentions. There are already several reports about the influences of commercial HAs on sludge AD (Azman et al., 2017; Liu et al., 2015; Li et al., 2019b). However, these findings are quite different and sometimes even contradict with each other. On the one hand, some of the studies reported the existence of HAs was harmful to AD process. For example, Xu et al. (2020) reported that commercial HAs enhanced the structural stability of organics in sludge and restricted their solubility. Ahmad et al. (2017) indicated high-concentration HAs inhibited hydrogenotrophic methanogens by more than 75% and Azman et al. (2017) showed that HAs with increased concentrations reduced hydrolysis efficiency by 40%. On the other hand, some works stated the presence of HAs was beneficial to AD process. For example, Li et al. (2019a) and Liu et al. (2015) reported that the presence of HAs improved sludge hydrolysis, and Ghasimi et al. (2016) showed that HAs had no effect on cumulative methane production.

Based on the recent published results, the mechanisms of HAs involved in the influences on sludge digestion can be roughly defined as four categories, namely binding with enzymes and substrates, electron shuttling, regulation to microbial communities, and complexation with metal ions (Li et al., 2019a; Xu et al., 2020). Thus, in this present study, we hypothesized that the effects of HAs on sludge AD are not only related to the contents of soluble HAs, but also to their structural characteristics, which shall explain these conflicting findings reported. Therefore, the aim of this current study was to explore the actual evolutions of HAs during sludge thermal hydrolysis, as well as their influences on sludge digestion. The changes of soluble HAs in concentrations and structural characteristics were investigated by gradually elevating hydrolysis intensity. Under HAs stresses with different concentrations and structural characteristics, the performances of sludge anaerobic fermentation for volatile fatty acids (VFAs) production were investigated to determine HAs inhibitions to hydrolysis phase, and acetate digestion for methane production were implemented to shed an insight on HAs influences to methanogenesis phase. Coupling effects of concentration and structural characteristics of HAs on their inhibition to hydrolysis were studied. Besides, correlation analyses between structural characteristics of HAs and their inhibitory effects on VFAs production were carried out.

## 2. Materials and methods

### 2.1. Waste activated sludge

WAS used as the substrate for AD was taken from the sludge storage tank of a local WWTP in Wuxi city, China. The fresh WAS was pre-concentrated before used (Liu et al., 2017). The concentrated WAS had pH of 6.5–7.5, total solids (TS) of 140–160 g/L, VS of 70–90 g/L, and SCOD of 130.0–140.0 mg/L. All measurements were conducted in triplicate.

### 2.2. Sludge thermal hydrolysis

Sludge thermal hydrolysis was performed in a thermal hydrolysis reactor with effective volume of 0.6 L. This reactor consisted of a control unit, electrical heating, cooling system, thermometers, pressure gage, and reducing valves. The data acquisition and control system were used to measure pressure and temperature. The prepared sludge samples of WAS mentioned above were diluted with deionized water to TS of 10% were firstly heated to the desired temperature, namely 40, 60, 100, 140, 160, 180, 200 and 220 °C, respectively. Then, sludge hydrolysis proceeded under the constant temperature for 30 min before the cooling

system was switched on. When the temperature dropped to 40 °C, the pressure reducing valve was open. Finally, all of the hydrolyzed sludge was taken out and centrifuged at 10,000 rpm for 10 min to separate the solid and the liquid.

### 2.3. Anaerobic fermentation for VFAs production

The experiments were conducted in 500 mL serum bottles. Each bottle was fed with substrate (glucose or sludge hydrolyzate) and seeding sludge to 250 mL at a volume ratio of 9:1. HAs used in the study were extracted from thermal hydrolysate. Dosages of HAs were 0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L, respectively. Then, pHs of all of the bottles were adjusted to  $10.0 \pm 0.2$  by diluted HCl and NaOH solutions. Oxygen in the headspace and the liquid was purged by N<sub>2</sub> gas for 15 min to maintain strict anaerobic condition. During the entire batch test, bottles were placed in a shaking incubator with a rotation speed of 130 rpm, temperature of 35 °C and pH of 10.0. Samples taken from bottles at certain intervals were analyzed. All the bottles were adjusted to pH of 10.0 and purged by N<sub>2</sub> gas for 15 min to maintain strict anaerobic condition after taking out the samples regularly every day. All the experiments were carried out independently in triplicates.

### 2.4. AD for methane production

The experiments were conducted in 500 mL serum bottles. Each bottle was fed with WAS and seeding sludge to 250 mL under a VS ratio of 5:2. The VS concentration of WAS and seeding sludge were  $20.88 \pm 0.78$  g/L and  $50.70 \pm 2.73$  g/L, respectively. HAs used in the study were extracted from thermal hydrolysate. Dosages of HAs were 0, 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 4.0 g/L, respectively. Then, all of the bottles were adjusted to pH of  $7.0 \pm 0.2$  by dilute HCl and NaOH solutions. Oxygen was removed by N<sub>2</sub> aeration for about 15 min. Bottles were placed in a shaking incubator with rotation speed of 130 rpm and temperature of 35 °C. Each bottle was connected to an external gas collection bag which was replaced daily. All the experiments were carried out independently in triplicates.

In order to explore the effect of HAs on methanogenesis, sodium acetate of 10 g/L was used as substrate for AD for methane production. The dosage levels of HAs were 0, 0.2, 0.4 and 0.6 g/L. The used bottles were the same as that mentioned above in VFAs production. pH was adjusted to  $7.0 \pm 0.2$ . Oxygen was removed by N<sub>2</sub> gas for about 15 min. Bottles were placed in a shaking incubator with a rotation speed of 130 rpm and temperature of 35 °C. Each bottle was connected to an external gas collection bag which was replaced daily. All the tests were carried out independently in triplicates.

### 2.5. Extraction of HAs

The freeze-dried and sieved samples were used to extract HA according to the method implemented by the International Humic Substances Society (IHSS) (Thurman et al., 1981). HCl of 6 mol/L was used to acidify the samples, including sludge and hydrolyzate, to pH of 1.0 to get the precipitate. NaOH of 6 mol/L was used to dissolve the precipitates to get the supernatant. Operations mentioned above were repeated to get the purified precipitates. The precipitates were further acidified by the mixed solution of 0.1 mol/L HCl and 0.3 mol/L HF (the volume ratio of 1:1), and then shaken for 12 h. Finally, the supernatants were discarded and the precipitates (HAs extracts) were washed for several times with ultrapure water to completely remove chloride ion. All the HAs samples were freeze-dried into the powder. The HAs obtained from WAS without THP and hydrolyzate of pre-treated sludge at 40, 60, 100, 140, 160, 180, 200 and 220 °C, were tagged as WNHAS, 40 °C HAs, 60 °C HAs, 100 °C HAs, 140 °C HAs, 160 °C HAs, 180 °C HAs, 200 °C HAs, and 220 °C HAs, respectively. The above-mentioned IHSS method was used to extract the HAs samples in solid phase and liquid phase respectively. Soluble HAs represented humic-like acids in the

liquid phase. The gravimetric method was used to quantify the contents of HAs in the samples (Lee et al., 1992). The loss part is equal to the amount of total HAs minus the sum amount of HAs in the liquid phase and HAs in the solid phase. Total amount of HAs refers to the HAs content in raw sludge that has not been thermally hydrolyzed. Each sample was analyzed in triplicate and the standard deviations of all analyses were less than 5%.

## 2.6. E4/E6 ratio

E4/E6 ratio is an important index indicating the structural characteristics of HAs and inversely proportional to the aromatic condensation. HAs with low E4/E6 ratio are difficult to be decomposed during AD (Chen et al., 1977; Tanja et al., 2017). To obtain the E4/E6 ratio, the absorbance at 465 nm and 665 nm of solutions of 30 mg extracted HAs in 100 ml 0.05 mol/L NaHCO<sub>3</sub> were recorded using a UV-VIS Spectrophotometer. The ratio of absorbance at 465 nm to that at 665 nm was defined as the E4/E6 ratio (X.W. Li et al., 2013). Each sample was analyzed in triplicate and the standard deviations of all analyses were less than 5%.

## 2.7. Excitation emission matrix (EEM) fluorescence spectra

All the supernatants of hydrolyzed sludge and extracted HAs samples were filtered by a filter membrane of 0.22 μm and then uniformly adjusted to the same concentration of about 200 mg/L before the EEM measure. EEM fluorescence spectra of tested samples with TOC of about 77.8 mg/L were analyzed with an F-7000 FL spectrophotometer produced by Hitachi. The excitation and emission wavelengths were gradually increased from 200 to 550 nm and 200 to 550 nm, respectively, in 5 nm increments. Organics in each sample could be divided into five groups according to the three-dimensional fluorescence region formed by the excitation and emission wavelengths. In general, peaks at shorter excitation wavelengths of <250 nm and shorter emission wavelengths of <380 nm are related to simple aromatic proteins, such as tyrosine (Region I and II). Peaks at excitation wavelengths of <250 nm and emission wavelengths of >350 nm are related to the fulvic acid-like organics (Region III). Peaks at intermediate excitation wavelengths of 250–280 nm and emission wavelength of <380 nm are related to soluble microbial byproduct-like material (Region IV). Peaks at excitation wavelengths of >280 nm and emission wavelengths of >380 nm are related to humic acid-like organics (Region V). Fluorescence regional integration (FRI) is a quantitative method to analyze fluorescence EEMs (Chen et al., 2003). Each sample was analyzed in triplicate and the standard deviations of all analyses were less than 5%.

## 2.8. Fourier-transform infrared (FTIR) spectra

All samples, the extracted HAs from sludge, were firstly freeze-dried before FTIR measure (Amir et al., 2010). The FTIR spectra of sample were obtained in the wavelength range of 4000–1000 cm<sup>-1</sup> on a Nicolet 6700 FTIR spectrometer (ThermoFisher Scientific, USA). Each sample was analyzed in triplicate and the standard deviations of all analyses were less than 5%.

## 2.9. Elemental analysis

Elemental composition was carried out to analyze C/N and C/H ratios of HAs samples. The data was obtained from an element analyzer (Elementar vario MICRO cube, Germany).

## 2.10. Analytical methods for conventional indexes

To measure COD, samples were first centrifuged at 8000 rpm for 10 min, after which they were filtered with 0.45 μm filters. The COD value was measured by the potassium dichromate method. TS and VS values of

sludge were calculated according to the gravimetric method. Conventional indexes, including COD, TS, VS were analyzed according to the standard methods issued by the State Environmental Protection Administration of China (2002). VFAs concentrations in the filtrate were measured by a gas chromatograph (GC-2010, Shimadzu, Japan) equipped with an auto injector (AOC-20i, Shimadzu, Japan). Samples filtered via the membrane of 0.22 μm were acidified by 3 M phosphoric acid and stored at -20 °C until further use. The total VFAs concentration was calculated by summing up each individual VFA (Liu et al., 2017). The acidification rate was obtained by dividing VFA concentration by substrate concentration, both of which were indicated by COD. The equivalents of acetate, propionate, butyrate/isobutyrate, valerate/isovalerate, caproate, heptanoate and caprylate were equal to 1.07, 1.57, 1.82, 2.04, 2.21, 2.34 and 2.44 mg COD/g, respectively. Hydrolytic protease was employed using the methods by Liu et al., 2015 in hydrolytic phase, and F<sub>420</sub>-reducing hydrogenase was based on the method used by Trevors., 1984 in the methanogenic phase, respectively. Each sample was analyzed in triplicate and the standard deviations of all analyses were less than 5%.

## 3. Results and discussion

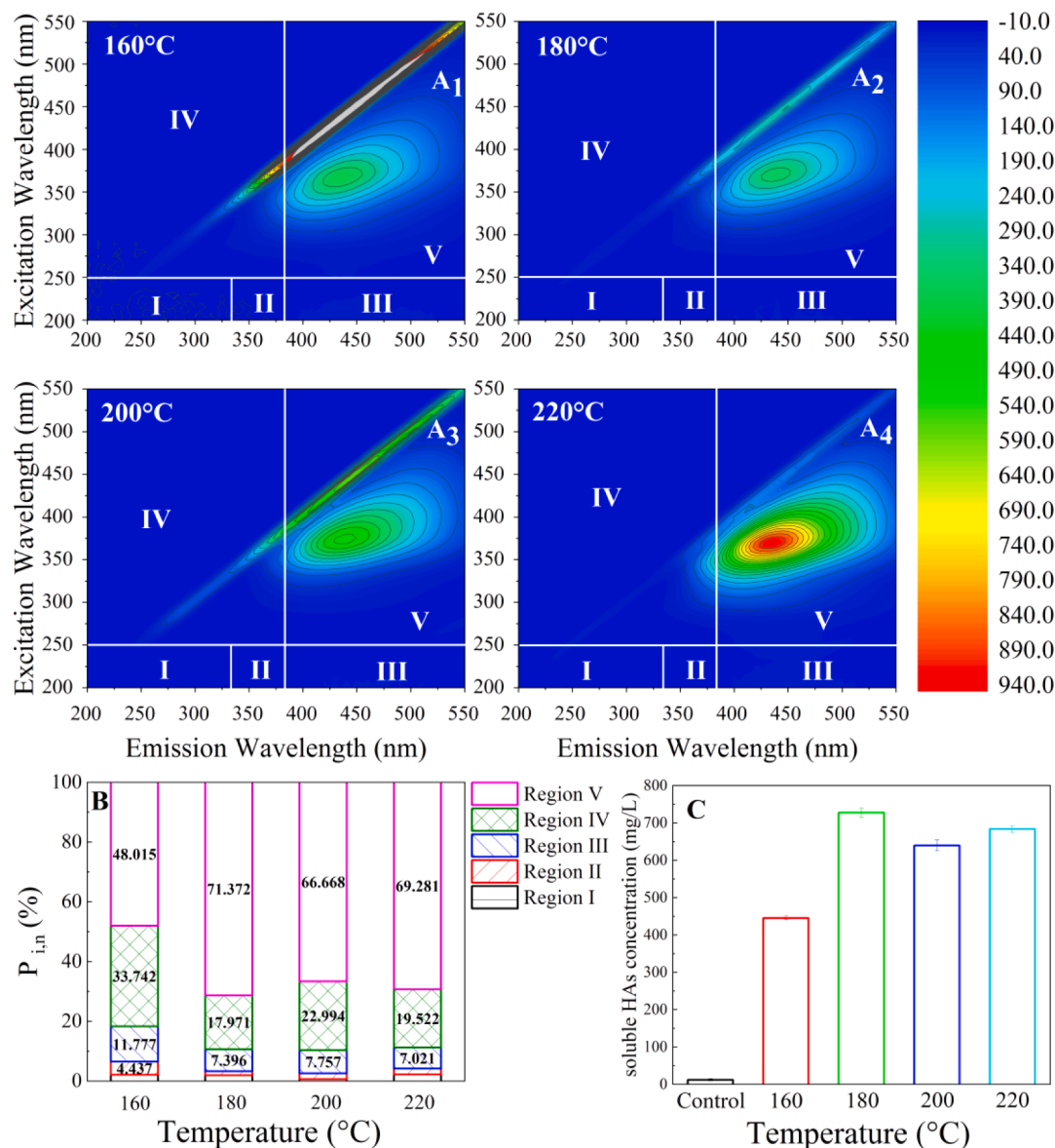
### 3.1. Enhanced release of HAs by thermal hydrolysis

#### 3.1.1. Improved concentration of soluble HAs in hydrolyzed sludge

High-temperature thermal hydrolysis strengthened HAs release from the sludge into the liquid, increasing soluble HAs concentration in thermal hydrolysate. According to Fig. 1A<sub>1</sub>-A<sub>4</sub>, FRI was performed to obtain the composition of the organic matter in the supernatant of thermal hydrolysis of the sludge. As shown in Fig. 1B, refractory components, mainly humic and fulvic acids, dominated the organics in hydrolysate. Li et al. (2016) also reported that alkaline pretreatment resulted in an increase of residual dissolved organic matter, which was mainly composed of humic substances (52.9%) and high molecular weight protein-like substances (49.9%). Moreover, as shown in Fig. 1C, HAs release was significantly strengthened as hydrolysis temperature was increased from 100 to 180 °C. The concentration of soluble HAs reached the maximum of approximately 727 mg/L at 180 °C and tended to decrease under a further enhancement of hydrolysis temperature. The content of soluble fulvic acids also changed with the increase of the hydrolysis temperature. Fulvic acids was converted to HAs via the abiotic processes during the pretreatment (Yang et al., 2016). Therefore, results indicated that it was important to adjust the temperature during sludge thermal hydrolysis to control the formation of refractory components.

#### 3.1.2. Distribution of HAs in hydrolyzed sludge

Although large amounts of HAs in sludge were released into the supernatant by thermal hydrolysis, most of the HAs still existed in the solid phase and kept at inert states. As shown in Fig. 2, almost all of the HAs in raw sludge were in the solid phase. With the increase of hydrolysis temperature, HAs in sludge tended to transform from the solid phase to the liquid phase, but their concentrations in liquid phase would no longer increase and even slightly decrease as the hydrolysis temperature reached a certain level, such as over 180 °C. Moreover, during the thermal hydrolysis, there appeared to be a loss in the total amount of HAs in sludge, which should be attributed to the larger amount of decomposed HAs than generated at excessively high temperatures. Wang et al. (2020) also reported HAs were transformed to other organics, such as fulvic acids, char or tar. Yang et al. (2016) also observed a mutual conversion between humic and fulvic acids during the thermal hydrolysis process. However, with the further increase of hydrolysis temperature, the content of HAs in solid seemed to keep stable relatively. Thus, it indicated that the chemical structure of HAs in the solid phase was rather stable and decomposition of HAs mainly happened in the liquid phase. Furthermore, it is worth to be noted that besides the



**Fig. 1.** EEMs of A<sub>1</sub>, A<sub>2</sub>, A<sub>3</sub> and A<sub>4</sub> (Region I, II: proteins, such as tyrosine; Region III: fulvic acid-like organics; Region IV: soluble microbial byproduct-like material; Region V: humic acid-like organics) and FRI of different thermal hydrolysis supernatant (B) as well as the concentrations of HAs extracted from hydrolyzate (C).

changes in concentrations, HAs structure also kept evolving with the increase of hydrolysis temperature, possibly making HAs more inhibitory to sludge digestion in particular with hydrolysis process during the AD (Liu et al., 2017; Xiao et al., 2019). Therefore, the results evidently indicated that thermal hydrolysis indeed induced the increase in concentrations and changes in structure of soluble HAs in sludge, which presumably leads to potential inhibitory impacts to sludge AD (Liu et al., 2015; Li et al., 2019a).

### 3.1.3. Response of HAs release to hydrolysis temperature

The amount of released HAs in the supernatant overall increased with the enhancement of hydrolysis temperature, but the production or release rate of the HAs changed greatly in different ranges of hydrolysis temperature. In order to elucidate the mathematical relationship between the hydrolysis temperature and the release of HAs, the range of low and medium temperatures was classified in the same group. As shown in Fig. 3, during thermal hydrolysis, the changes of dissolved HAs concentration presented evidently linear correlation with increase of the hydrolysis temperature ( $p < 0.05$ , \*). When the temperature was below 140 °C, the concentrations of soluble HAs gradually increased with the

enhancement of hydrolysis temperature and their coefficient ( $k$ ) was 1.2067. When the temperature increased from 140 to 180 °C, HAs concentration sharply increased and the rate coefficient ( $k$ ) was rapidly raised to 14.06. However, further enhancing hydrolysis temperature, the release rate of HAs started to slow down, possibly corresponding with HAs conversions into other substances through decomposition and condensation. Current thermal pretreatments for sludge digestion could be roughly divided into moderate and high temperature hydrolysis. The latter has already been widely used in full-scale projects under temperatures of 140–180°C. Thus, the results further confirmed the significance of focusing on the potential impacts of HAs release and conversion on sludge hydrolysis, especially in the range of 140–180°C.

## 3.2. Altered chemical structure of HAs during thermal hydrolysis

### 3.2.1. Changes in aromatic condensation

Aromatic condensation of HAs could be used to positively characterize their contents of hydrophobic portions with high molecular weight, such as aromatic acids and phenol. Liu et al. (2015) reported HAs with high hydrophobicity could improve solubilization of sludge

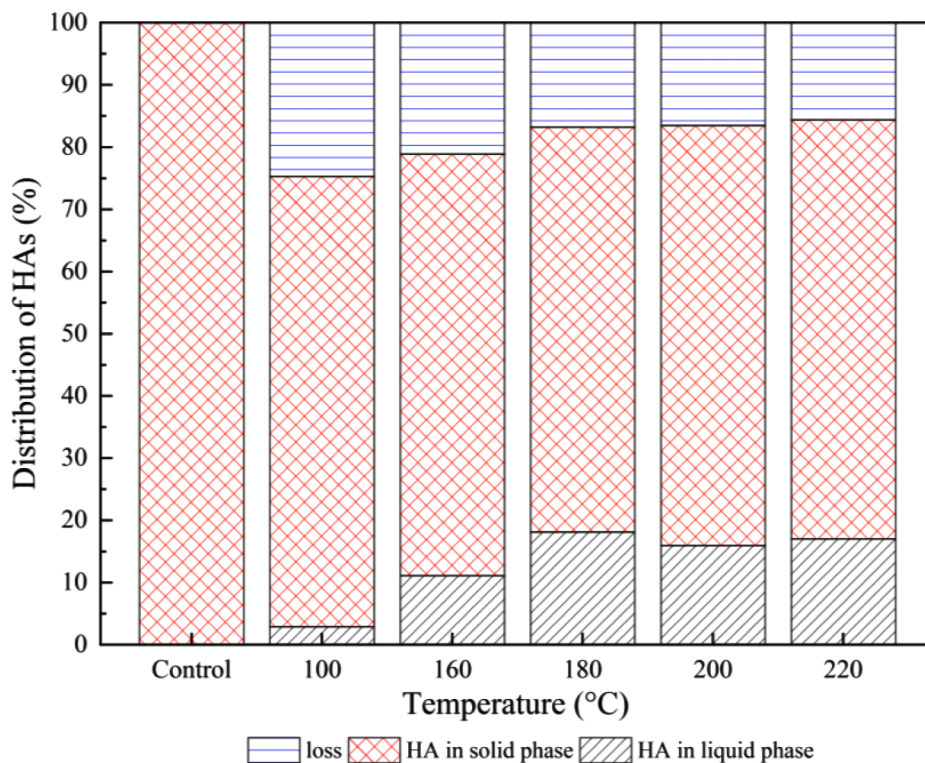


Fig. 2. HAs distribution in liquid and solid phases at different hydrolysis temperatures.

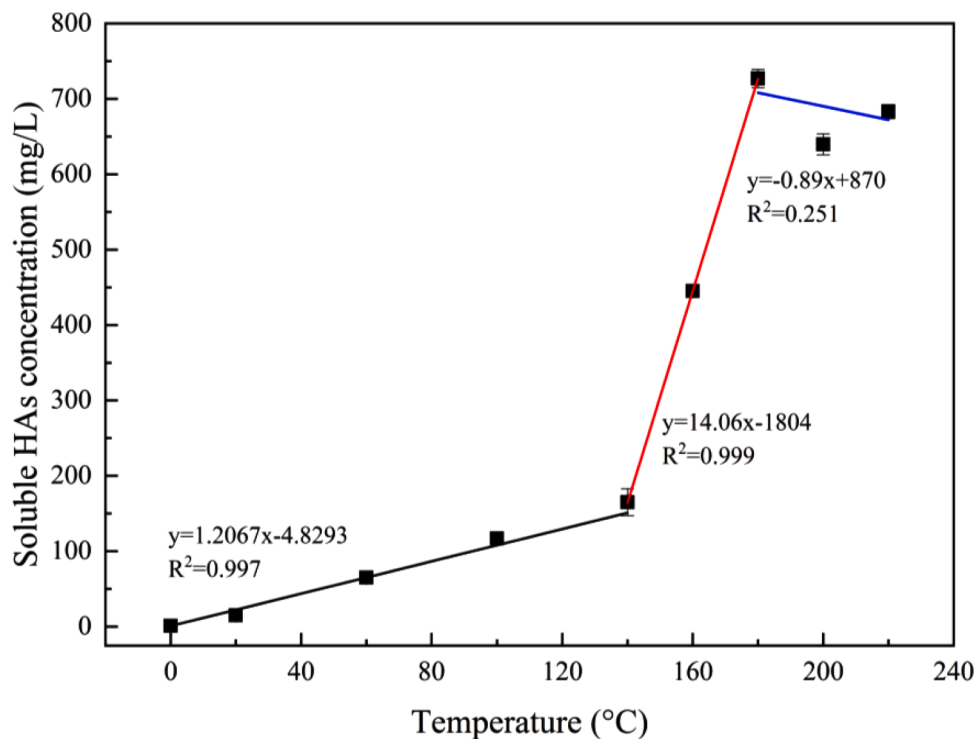


Fig. 3. Relationships between thermal hydrolysis temperature and the concentrations of soluble HAs.

particulate organic matter, just like the function of surfactant, by accelerating the dissolutions of organic compounds in sludge and maintaining the activity of hydrolase. Moreover, it also reported that aromatic condensation of HAs was positively related to their redox potential of the quinone groups (Li et al., 2019a).

Aromatic condensation of HAs is inversely proportional to the ratio

of E4/E6 (Chen et al., 1977). HAs with low E4/E6 ratio are difficult to be decomposed during AD (Li et al., 2013). As shown in Fig. 4, thermal hydrolysis at medium or low temperature, such as 100 °C, resulted in the decrease of aromatic condensation degree of HAs in sludge, while high temperature, such as 160–200 °C, caused the enhancement of HAs aromatic condensation. Comparing with WNHAs (the control), a slight

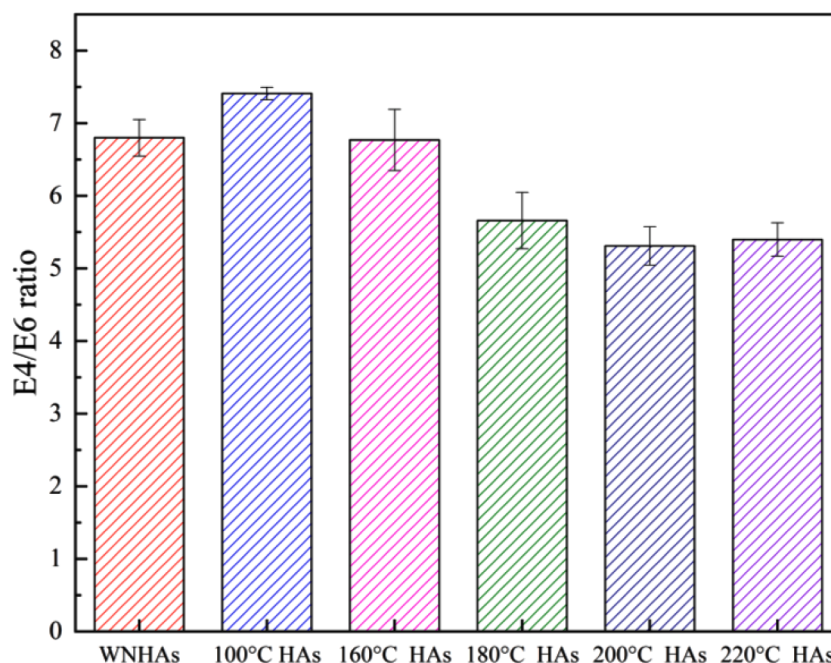


Fig. 4. E4/E6 ratios of the HAs extracted from sludge hydrolysate with different temperature, namely 100 °C (100 °C HAs), 160 °C (160 °C HAs), 180 °C (180 °C HAs), 200 °C (200 °C HAs), 220 °C (220 °C HAs), respectively, as well as the control (WNHAs).

increase of E4/E6 ratio happened in the HAs extracted from the hydrolyzed sludge under 100 °C (100 °C HAs), which was likely ascribed to the breakage of the aromatic rings during hydrolytic process (Rodriguez et al., 2016). However, with further increase of temperature, E4/E6 ratio of HAs in the hydrolyzed sludge tended to decrease substantially, and the aromatic condensation of sludge HAs reached the maximum at the temperature of 200 °C. Therefore, the results indicated thermal hydrolysis induce the changes of HAs structure and make the HAs more aggressive to sludge digestion. However, most of recent publications merely focus on the influences of HAs concentration during sludge digestion, which is likely to underestimate the effect of HAs.

### 3.2.2. Changes in elemental composition

Elemental composition is also an important indicator of HAs biochemical activity, which can be used to analyze the change law of HAs structure during sludge thermal hydrolysis and predict their influences on AD. For example, Hernandez et al. (1988) reported that nitrogen content in HAs was positively relative to the contents of amino-acids and microorganism tissue which had good bioavailability. As shown in Table 1, the content of nitrogen in HAs was sharply reduced by the increased temperature. Therefore, the results indicated that with the enhancement of thermal hydrolysis temperature, HAs not only underwent changes and/or migrations of functional group and an increase in aromaticity, but also in essence, the overall bioavailability of HAs decreased. Moreover, the result could be further confirmed by the decreased H/C ratio in HAs with the increase of sludge hydrolysis temperature (as shown in Table 1). García et al. (1992) and Fernandes et al. (2015) reported that the ratio of H/C (<1) represented the high

aromatic condensation. Furthermore, the changes in elemental composition reflected the variations of functional group structure. The C/N ratio was negatively relative to the content of aliphatic-N and positively relative to that of heterocyclic structures (Hernandez et al., 1988; Li et al., 2014). As shown in Table 1, the ratio of C/N rapidly increased with the enhancement of hydrolysis temperature mainly due to the formation of N-substituted glycosylamine by the condensation reactions between amino acids and sugars. Therefore, the analysis of elemental composition indicated that as the temperature increased, the HAs structure tended to be stable and the biodegradability would decrease.

### 3.2.3. Changes in functional groups

The functional groups of HAs in pretreated sludge were also changed during thermal hydrolysis. The characteristics of infrared spectra of the soluble HAs were shown in Fig. 5. Comparing with the control and pretreated sludge under 100 °C, soluble HAs extracted from the hydrolysate of the pretreated sludge under 160 °C (160 °C HAs) tended to have fewer functional groups which were mainly composed of three-bond functional groups (C≡C) and concentrated at near wavenumber (Amir et al., 2010). As temperature increased, the types of functional groups increased and the groups were gradually dominated by single bond functional groups. The spectra of the soluble HAs extracted from pretreated sludge under 180 °C (180 °C HAs) showed a high band intensity at 1650 (amide I), 1230 and 1260  $\text{cm}^{-1}$  (C—N stretching, carboxyl group, O—H bending). The results indicated high contents of amides, ethers and esters, that is, the oxidation of this HAs was relatively low (Tristan et al., 2018). As the hydrolysis temperature continuously increased up to 200 and 220 °C, the amount and intensity of the peaks in spectra obviously increased, possibly because of decomposition reactions. Therefore, the results indicated that as the hydrolysis temperature increased, the abundance of functional group types in the HAs extracted from the pretreated sludge decreased firstly and then increased, simultaneously accompanying with the changes in the total amount and structure of functional groups.

Table 1  
Elemental composition of HAs extracted from pretreated sludge.

Samples	N (%)	C (%)	H (%)	C/N	C/H	H/C
WNHAs	10.66	45.33	7.27	4.25	6.23	0.16
100 °C HAs	8.65	46.66	6.19	5.39	7.54	0.13
160 °C HAs	8.06	50.99	5.89	6.33	8.65	0.12
180 °C HAs	7.72	57.99	6.75	7.51	8.59	0.12
200 °C HAs	7.36	58.80	6.43	7.99	9.15	0.11
220 °C HAs	5.69	58.96	6.51	10.37	9.06	0.11

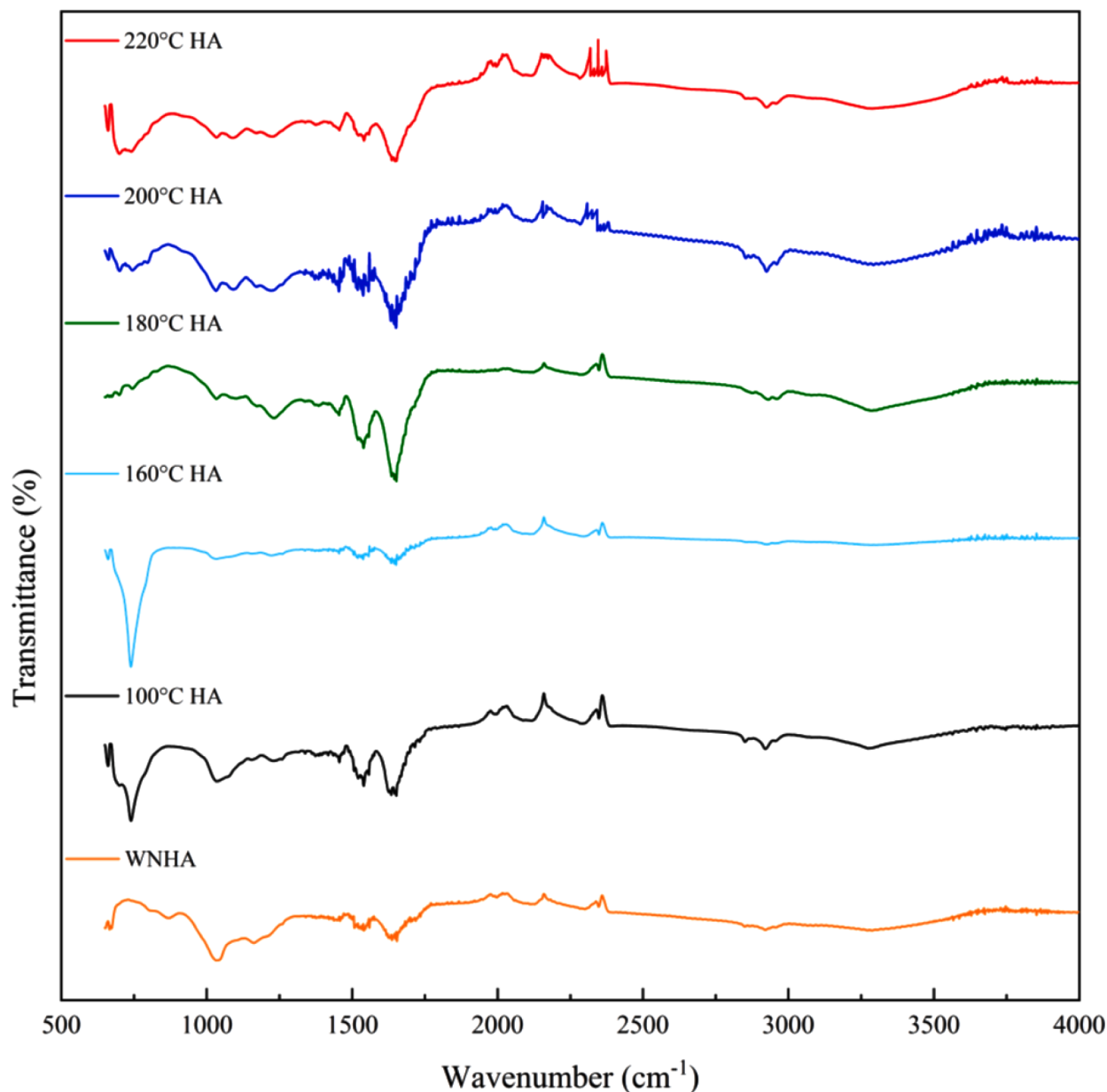


Fig. 5. FTIR of HAs extracted from pretreated sludge hydrolysate under different temperatures.

### 3.3. Influences of HAs release on sludge AD

#### 3.3.1. Inhibition of HAs with enhanced concentration to sludge fermentation for VFAs production

Sludge fermentation for VFAs production were severely inhibited by the enhancement of soluble HAs concentrations. As shown in Fig. 6A, when the HAs concentration was just elevated by 0.2 g/L, the total VFAs concentration decreased to the half of that of the control, from 9800 to 4800 mg/L. Moreover, the total VFAs concentration presented a sharp decrease over the further increase of HAs concentrations. The results obtained in this study differ from that observed reports. For example, Liu et al. (2015) reported VFAs production from sludge hydrolysis was accelerated due to the increase of HAs concentration. However, it was interestingly found that the humic substances reported to be inhibitory were mainly from the extracts of soil, sludge, landfill leachate, and maize etc., whereas those beneficial to AD were mainly fossil or commercial (Liang et al., 2021). The influences of HAs on sludge hydrolysis are not only related to their concentrations, but also related to their structure.

As mentioned above, the mechanisms of influences of HAs on sludge digestion mainly include bundling, electron shuttling, microbial regulation, and metal complexation (Li et al., 2019a). As shown in Fig. 6B, the increase of HAs concentration presented no obvious impact on the composition of VFAs production from anaerobic fermentation of sludge. The dosage of HAs increased from 0 to 1.0 g/L, acetate kept being the predominant VFAs component in each group and accounted for 53%–85%, followed by propionate, which accounted for 5%–10%. Isobutyrate, butyrate, isovalerate, valerate, caproate, heptanoate were almost around 4%, followed by a few caprylate. Thus, the results indicated the increase of HAs concentration presumably had little influence on the composition of the microbial community and its metabolic pathways (Li et al., 2021; Azman et al., 2017). Meanwhile, given that the content of heavy metal in the sludge used in this study was very low, the role of HAs in complexation with metal ions was limited. Furthermore, via electron transfer, the hydrolysis of organics in sludge could often be enhanced (Liu et al., 2013; Liu et al., 2015). Therefore, the main inhibition mechanism of HAs on sludge hydrolysis should be the bundling of HAs with functional enzymes and substrates.

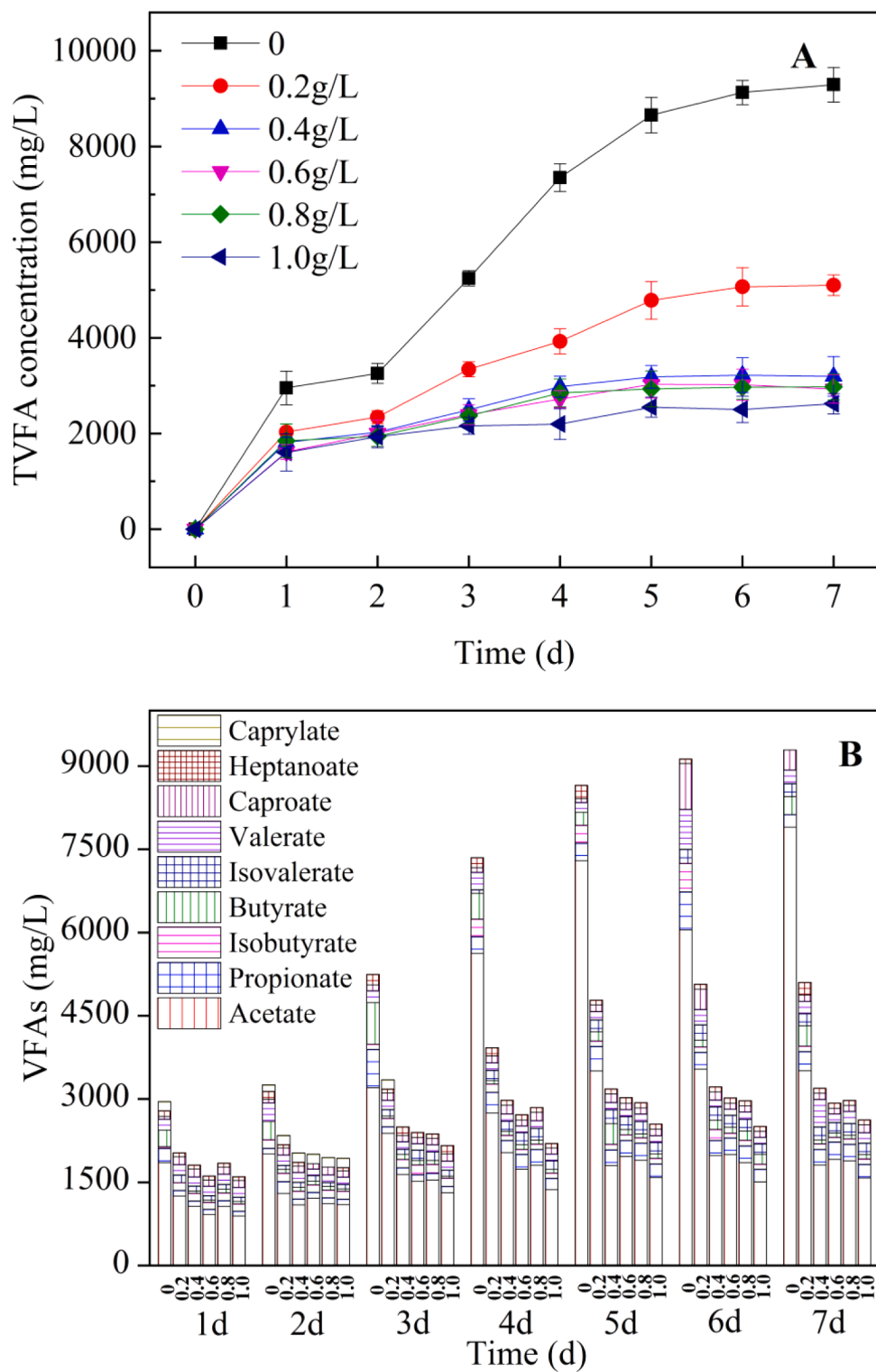


Fig. 6. Yield (A) and composition (B) of VFAs produced from sludge fermentation under different HAs concentration.

3.3.2. Inhibition of HAs with altered structure to fermentation for VFAs production

The inhibition of HAs to sludge hydrolysis was related to not only their concentrations, but also their structural characteristics. As shown in Fig. 7, their effects on acidification rate from glucose fermentation varied greatly between HAs with the same concentrations but obtained from different sludge hydrolysates. Except for the HAs extracted from 160°C hydrolyzed sludge, all of other HAs presented inhibitory effects

on glucose fermentation and their inhibition became more acute with the increase of HAs concentrations. Though the half-inhibitory concentrations of each HAs were different, most of the HAs were around 0.4 g/L.

However, significant differences existed in inhibition degree between different HAs to glucose fermentation. As shown in Fig. 7, under the same concentrations, HAs extracted from the raw sludge (WNHAs) and medium-temperature pretreated sludge (100 °C HAs) seemed more



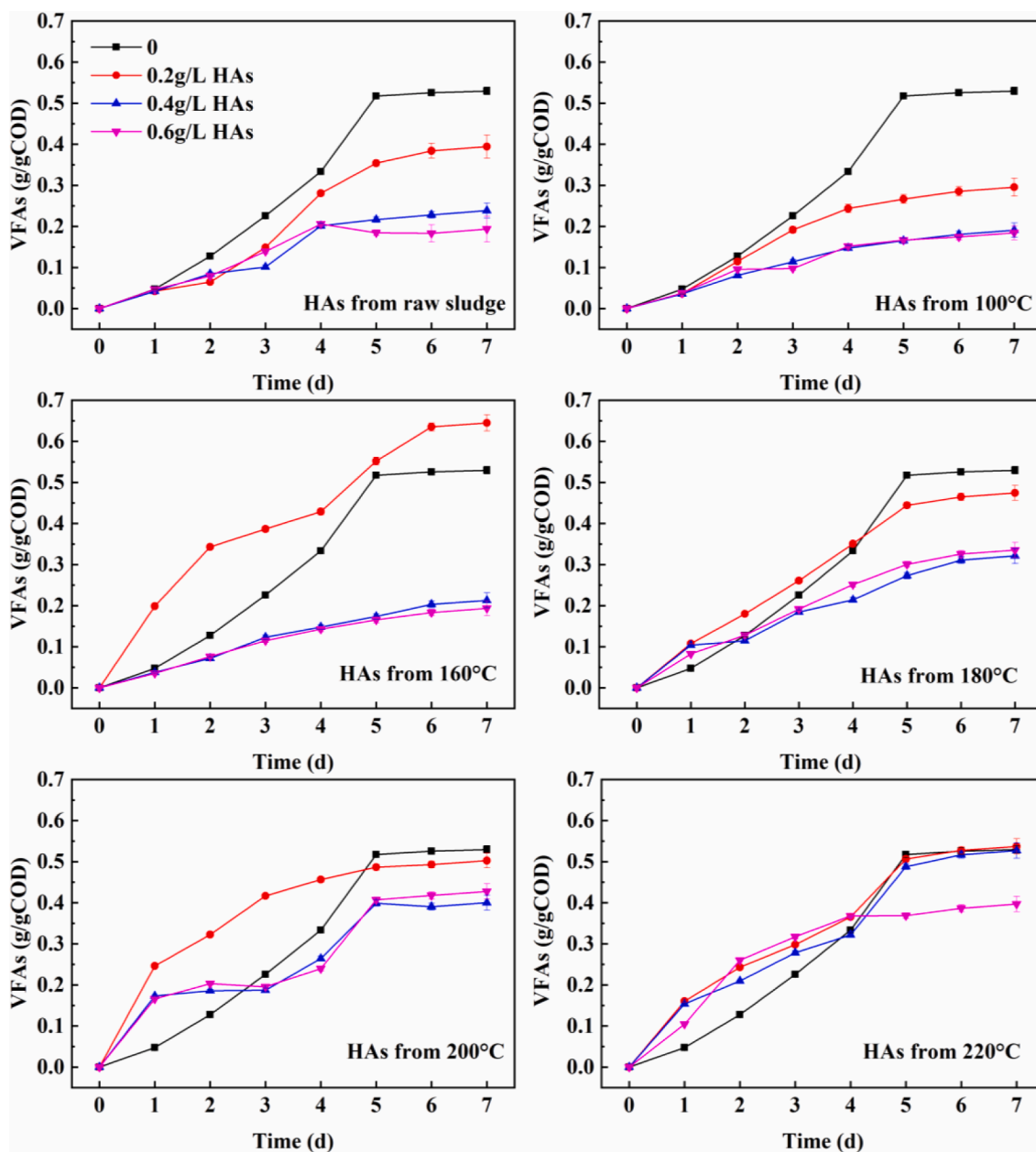


Fig. 7. Effects of HAs with different structures on acidification rate from glucose fermentation, in which the HAs were extracted from sewage sludge (A, WNHA) and pretreated sludge hydrolyzed under 100 °C(B, 100 °C HAs), 160 °C(C, 160 °C HAs), 180 °C(D, 180 °C HAs), 200 °C(E, 200 °C HAs) and 220 °C(F, 220 °C HAs), respectively.

inhibitory than other HAs extracted from high-temperature pretreated sludge samples, namely 160 °C HAs, 180 °C HAs, 200 °C HAs, and 220 °C HAs. Especially, at low concentration of 0.2 g/L, the addition of 160 °C HAs improved VFAs production from glucose fermentation. The observed phenomenon possibly was attributed to the enhanced stability of HAs structure during the increase of hydrolysis temperature.

Moreover, correlation analysis was implemented to quantify the influences of HAs structural characteristics on their inhibition to hydrolysis of organics. Considering the several main structural characteristics aforementioned, as shown in Table 2, the influence of HAs structural characteristics could be neglected at low concentration of 0.2 g/L, but become more and more significant with the increase of HAs concentration. E4/E6 and C/N ratios presented significant correlation ( $p < 0.05$ , \*) with the inhibition of hydrolysis of organics when HAs concentration was 0.4 g/L. However, as HAs concentration increased, some structural features, such as C/H of HAs, always showed no significant correlation

with HAs inhibition to the hydrolysis of organics. Thus, the results indicated that release of HAs by thermal hydrolysis inhibited biodegradation of the organics during sludge digestion. The performance of the AD process was evidently related to HAs concentration and structural characteristics. And within a certain concentration range, quantitative relationships between HAs structural characteristics and the inhibition to the biodegradation of organic matter are demonstrated.

### 3.3.3. Influences of released HAs on methanogenic AD

Sludge AD for methane production was promoted by HAs at low concentration and inhibited at high-concentration. As shown in Fig. 8, methane production was firstly promoted when HAs concentration in the reactors was less than 1.0 g/L. However, the enhancement in  $\text{CH}_4$  production seemingly reached the peak at the concentration of HAs of about 0.8 g/L and then tended to decline. Moreover, when HAs concentration further increased to 2.0–4.0 g/L, sludge AD was heavily

**Table 2**

Correlations between HAs structural characteristics and its inhibition to VFAs production during glucose fermentation.

Concentration (g/L)	Factor	Correlation (P)	Fitting formula
0.2	E4/E6	0.359	No significant correlation
	C/N	0.325	
	C/H	0.100	
0.4	E4/E6	0.02*	$y = 3.5 - 0.90x + 0.06x^2, R^2 = 0.82$
	C/N	0.01*	$y = 0.5 + \frac{0.19}{1 + \exp(x - 7.7)/0.3}, R^2 = 0.99$
0.6	C/H	0.187	No significant correlation
	E4/E6	0.002*	$y = 0.18 + \frac{0.16}{1 + \exp(x - 5.4)/0.4}, R^2 = 0.99$
	C/N	0.033*	$y = \frac{0.19}{1 + \exp(x - 0/75)/0.018}, R^2 = 0.99$
	C/H	0.100	No significant correlation

inhibited and the cumulative methane production was only about half of that of the control group. As shown in Fig. 8, the daily CH<sub>4</sub> production in each test sharply increased until they reached the maximum, but the tests at low HAs concentration (0–0.6 g/L) reached their peaks four days earlier (Day 4) than other tests with HAs concentration of 0.8–1.0 g/L. With the further increase of HA dosage up to 2.0–4.0 g/L, the daily methane production reached the maximum on Day 11 and HAs caused a lag phase in daily methane peaks. Therefore, the results indicated that the mechanisms of the influences of HAs on sludge AD were likely multiple and could be roughly divided into two distinct types, one boosting methane production and the other inhibiting methane methanogenesis. For example, Azman et al. (2017) reported HAs could inhibit sludge AD by binding hydrolase, but Li et al. (2019a) found HAs could promote sludge AD by facilitating electron transfer. Under low concentrations, the contribution of HAs to sludge AD was greater than inhibition, whereas under high concentrations, vice versa.

Methane production from sludge AD included hydrogenotrophic and acetoclastic methanogenesis. The presence of HAs at low concentrations evidently promoted acetoclastic methanogenesis. As shown in Fig. 9, almost at all levels of HAs concentrations, the processes of methane production from acetate digestion were clearly boosted, and methane production was enhanced significantly as the content of HAs increased.

However, some divergences among different HAs were also observed. The HAs from raw sludge and sludge pretreated at a medium temperature showed stronger promotion effects to methanogenesis than that of HAs from sludge pretreated at a high temperature. For example, HAs extracted from hydrolyzed sludge under 160 °C (160 °C HAs) improved biogas production by nearly 200%, while 200 °C HAs did not evidently strengthen methane production. Liu et al. (2015) found the introduction of commercial HAs during sludge digestion affected methane production, namely no noticeable influence on hydrogenotrophic methanogenesis but a clear inhibition to acetoclastic methanogenesis. The inhibitory effect was closely related to the characteristics of the commercial HAs. The mechanisms of influences of HAs on methanogenesis were mainly contributed to their competition with some key enzymes for electrons. Therefore, the different results observed in this study possibly are linked to that fact that the structural characteristics of HAs extracted from sludge determined their ability to gain and give electrons, which was presumably different from the characteristics of the commercial HAs.

### 3.3.4. Influence of released HAs on enzymatic activity

The increase of HAs concentration inhibited the activity of hydrolytic protease, but promoted the activity of the key enzymes for methanogenesis. As depicted in Fig. 10, after about 5 days operation at HAs concentration of 0.6 g/L, the activity of proteinase was decreased by 20%, while the activity of co-enzyme F<sub>420</sub> was increased to 119% in the reactors for anaerobic digestion of the sludge. HAs are defined as a cluster of organic compounds with different molecular sizes. HAs with large molecules might be rejected by cell structure and accumulated outside the cell of the fermenting bacteria, inhibiting the activity of extracellular hydrolase through binding (X.W. Li et al., 2013; Fernandes et al., 2015), while HAs with small molecules could pass through the cell membrane and facilitate in electron transfer to increase the activity of methanogenesis enzymes. The statement could also explain the observed phenomenon of influences of HAs on sludge AD. At high HAs concentrations, extracellular hydrolase possibly became inactivated by combining with HAs that abundantly existed outside of microorganism cells, while inside of the cells, due to the saturation of HAs, their contributions in electron transfer were presumably no longer increased with their concentrations. However, at relatively low concentrations, the inhibitory effect of HAs to extracellular enzymes was limited, which could be readily offset by their contributions on promotion of methanogenesis via electron transfer.

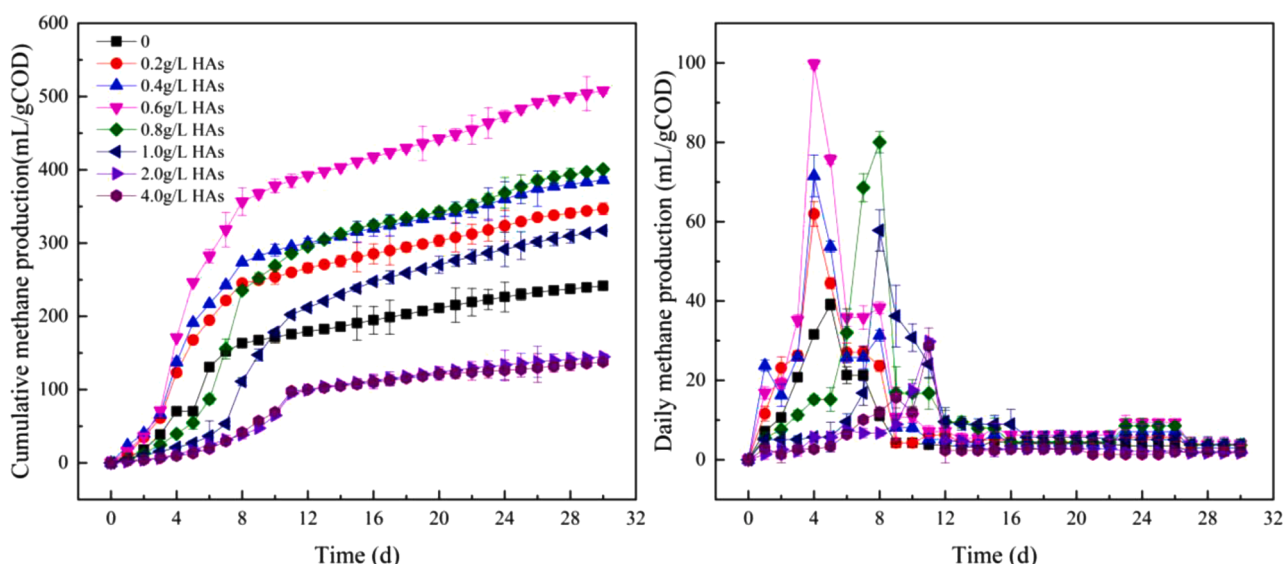


Fig. 8. Effects of HAs on the cumulative methane production and daily methane production from sludge anaerobic digestion.

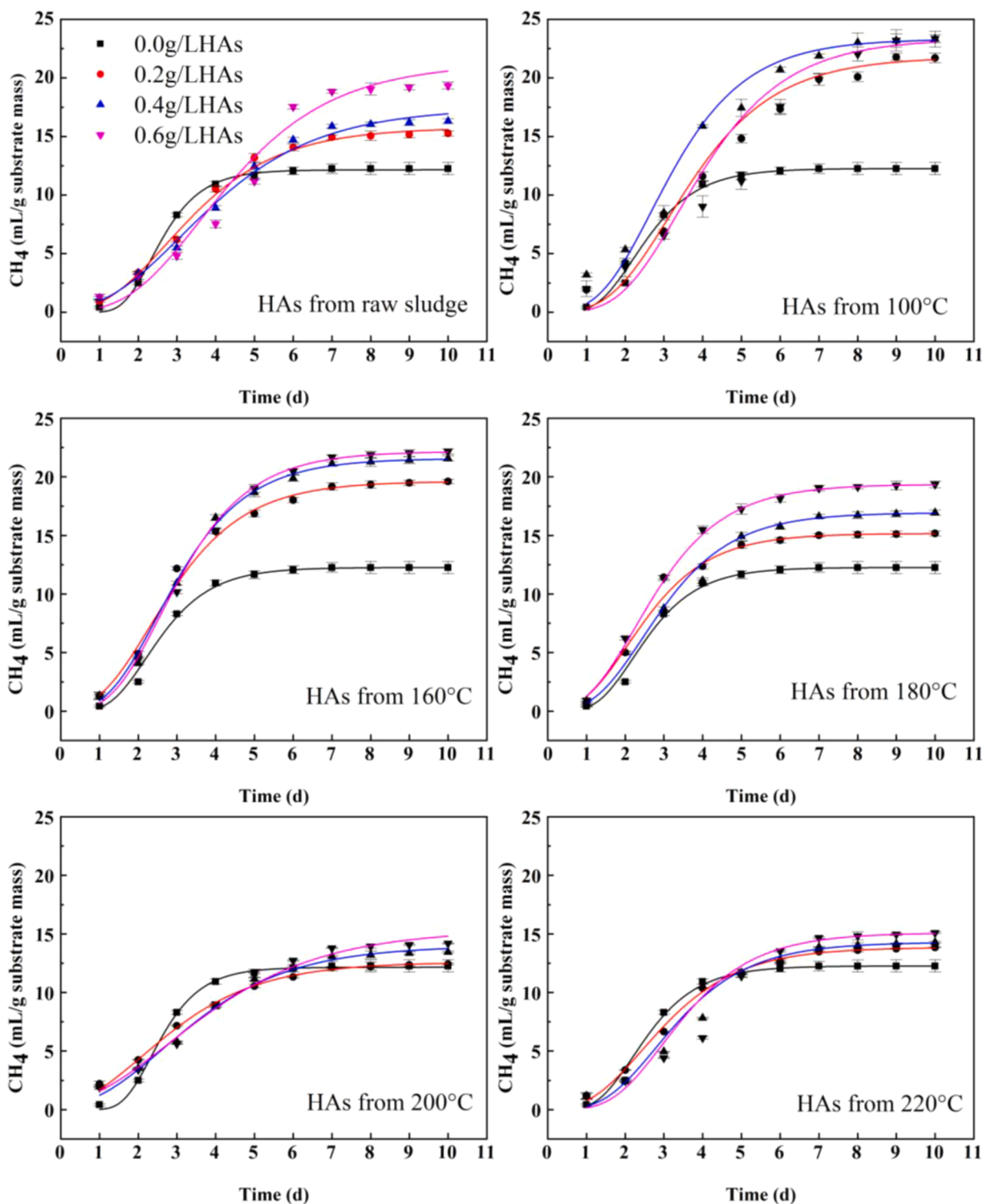


Fig. 9. Effect of HAs from hydrolyzed sludge under different temperatures on methanogenesis by modified Gompertz model.

### 3.4. Mechanisms analysis

The effect of HAs on sludge AD is the result of a combination of multiple mechanisms, include bundling, electron shuttling and microbial regulation. Based on the results obtained in this paper, it can be seen that HAs under low concentration could bind the dissolved organic matter and extracellular enzymes, reducing the sludge acidification efficiency. However, the cell walls of bacteria would prevent the macromolecular HAs passing through the cells, thus, the inhibition on

intracellular methanogenesis enzymes can be ignored. Although HAs with small molecule could pass through the cell membrane, the effect of binding enzymes was not significant, since the binding of HAs to enzymes acted like that of cellulose. Furthermore, HAs with small molecule participated in electron transferring, enhancing the activity of methanogenesis enzymes. Therefore, HAs under low concentration would inhibit VFAs production but improve methane production. However, HAs under high concentration could inhibit both acidification and methanogenesis. The former inhibition was caused by the bundle of

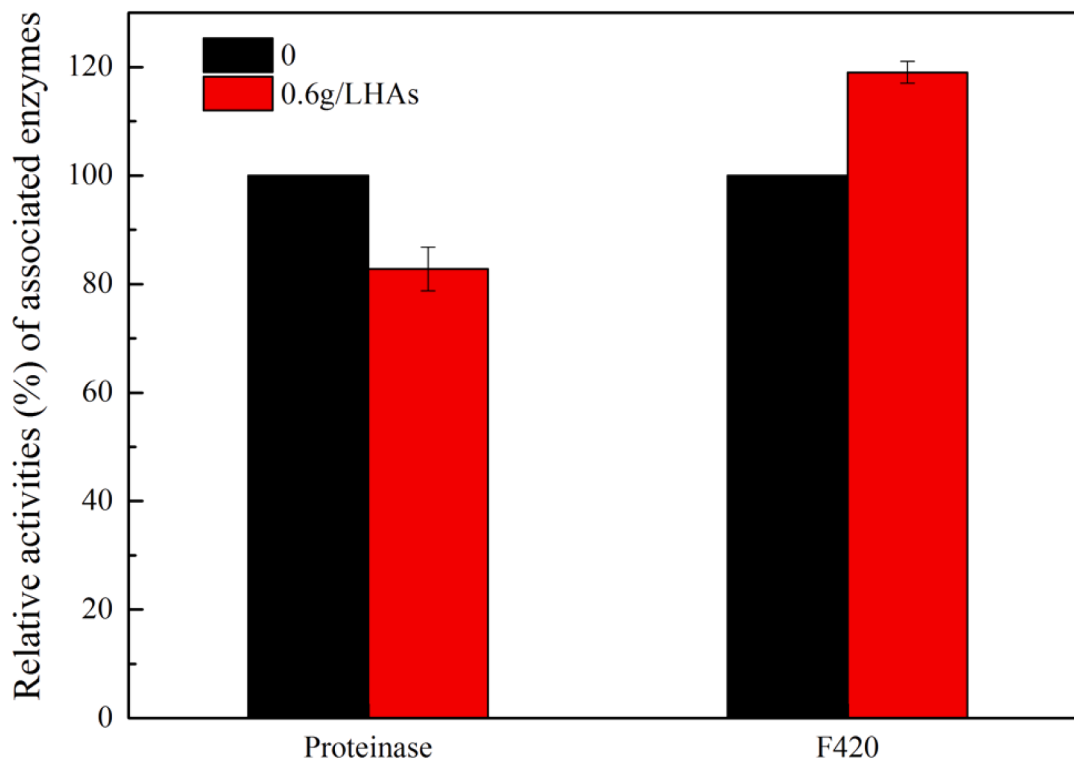


Fig. 10. Relative activities of proteinase and co-enzyme F420 on Day 5 at HAs concentration of 0.6 g/L.

HAs to substrates and enzymes, and the latter inhibition was probably related to the bunch of HAs to methanogens and dissolved organic matter during AD.

#### 4. Conclusions

HAs release and their influences on sludge AD with THP seemingly are underestimated, which are likely to be the causes to low methane yield. Results of this study presented an insight into the evolutions of HAs in sludge during THP, and proposed a new concept of synergistically interfering the AD via HAs concentration and structural characteristics. The following remarks are concluded:

- (1) HAs release from sludge can be accelerated with the increase of hydrolysis temperature. The concentration of soluble HAs substantially increased, from 8 mg/L in raw sludge to 727 mg/L in the pretreated sludge hydrolyzed at 180°C.
- (2) Chemical structure of HAs was severely altered during sludge THP. HAs extracted from sludge hydrolyzed at 200°C presented the largest aromatic condensation degree. Functional groups of 160°C HAs were mainly composed of unsaturated bonds, but as hydrolysis temperature raised, bonds in the functional groups began to break and single-bond substances formed.
- (3) Acidification rate was inhibited by over 50% with 0.4 g/L of HAs. Besides the concentration increases, changes of HAs structural characteristics also play an important role on their influences on sludge digestion. In particular, the E4/E6 and C/N ratios of HAs had a significant correlation with the inhibition to VFAs production during sludge anaerobic fermentation.
- (4) Sludge AD for methane production was promoted by HAs at low-concentration (below 1.0 g/L) and inhibited at high-concentration (over 2.0 g/L), and the extent of the effect was also closely relative to the structural characteristics of HAs. The increase of HAs concentration in sludge AD inhibited the activity of hydrolytic protease, but promoted the activity of the key enzymes for methanogenesis. The activities of proteinase and co-

enzyme F420 were decreased by 20% and increased by 19%, respectively, with HAs at 0.6 g/L for 5 days.

- (5) Due to the rate-limiting step was methanogenesis in the AD process of pretreated sludge by thermal hydrolysis, HAs concentration was recommended at low level, for example around 1.0 g/L, to accelerate or not limit methanogenesis.

#### Declaration of Competing Interest

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

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2021.117310](https://doi.org/10.1016/j.watres.2021.117310).

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