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Dynamics of humic substance composition during anaerobic digestion of excess activated sludge



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ARTICLE INFO	A B S T R A C T
Keywords:	Inhibition by humic substances (HSs) on anaerobic digestion of excess activated sludge is one of the limitations
Humic substances Excess activated sludge Anaerobic digestion Humic acid Fulvic acid Inhibition reverse	for converting more organics into biogas. In order to counteract the inhibition from HSs, the present study was initiated to understand the dynamic changes (content and structure) of HSs during the anaerobic digestion of synthetic and real sludge. For the first time, the present work studied the dynamics of HSs separately in the liquid and solid phases. These observations on HSs conversions by potentiometric titration, UV–Vis, FTIR, and two- dimensional infrared spectra, confirmed that the dynamic changes of HA and FA compositions were caused by losing aliphatic moieties and enriching aromatic moieties in the structural compositions. This changes increased the humification degree, aromaticity, and the amounts of oxygen-containing functional groups. Based on the observations of the all write the inhibition offerst ware dimensional

1. Introduction

In wastewater treatment plants, part of the organic energy is contained in the excess activated sludge (EAS) which carries up to $14.7 \text{ kJ}(\text{g COD})^{-1}$ (Logan, 2008) in theory. In practice, the energy in the EAS can be recovered via anaerobic digestion (AD) in the form of biogas (Hao et al., 2017a; Zhen et al., 2017). However, the AD process can extract hardly more than 50% of the total organic energy contained in the EAS due to various interferences (Weemaes et al., 2000; Raposo et al., 2012).

Humic substance (HSs) have been identified to interfere with the anaerobic digestion processes and compromise the biogas production particularly by negatively influencing the hydrolysis and the methanogenesis (Zhou et al., 2014; Liu et al., 2015; Azman et al., 2017; Li et al., 2019a). Thus, HSs along with ammonia, sulfide, and heavy metals have been listed as inhibitors to the AD (Chen et al., 2008; Azman et al., 2015). Indeed, HSs are major organic constituents of EAS and account for up to 6–20% of the volatile suspended solid (VSS) (Li et al., 2019b). HSs, based on their aqueous solubility, are usually separated into three categories, humic acid (HA), fulvic acid (FA), and humin (Davies and Ghabbour, 1998; Lipczynska-Kochany, 2018). Generally, they are derived from the residues of dead plants, animals, microorganisms, and their degradation products. The genesis of HSs as well as their structure are still under debate. Recently, it has been proposed that HSs consist of

supramolecular associations of heterogeneous and rather small molecules (Lipczynska-Kochany, 2018). In the process of sewage treatment, they are adsorbed onto the activated sludge flocs (Esparza-Soto and Westerhoff, 2003). In comparison with lignocellulose, HSs contain more active functional groups, especially carbonyl and carboxyl groups which could be involved in various physicochemical interactions (Ho and Ho, 2012; Liu et al., 2015). For this reason, several hypotheses have been proposed to explain the inhibitory effect of HSs on AD which include (a) binding to hydrolytic enzymes or hydrolytic bacterial cell and (b) competition for electrons (Fernandes et al., 2015; Azman et al., 2017; Li et al., 2019a).

Thus, in order to alleviate the inhibitory impact of HSs on AD several strategies have been proposed and tested, including (a) pretreatment to enhance the hydrolysis efficiency, (b) HSs removal to avoid the inhibition effect, and (c) additives (enzymes and cations) to shield the inhibition effect (Azman et al., 2015). Considering the energy consumption and practical feasibility, the *in-situ* dosage of inorganic cations or enzymes to passivate the active functional groups of HSs and thus shield the inhibition effect seems to be the only applicable strategy (Azman et al., 2015).

However, both the precise amount of cations or enzymes which are required to dose to alleviate the inhibition of HSs and the experimental protocols to determine it in practice on a real plant are still not consistent and subject to debate. That is because currently a critical HSs

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concentration presenting inhibitory effect cannot be defined from the reported results due to their complex structure and functional groups (Stevenson, 1994). Although some works tried to discover the dynamics of HSs in AD, most of them were conducted by adding external HSs in order to evaluate the inhibitory effect (Martinez et al., 2013). However, this kind of commercial HSs products may not be representative of the HSs actually present in the AD (Lipczynska-Kochany, 2018).

For these reasons, this experimental research was initiated to study the dynamics of autochthonous HSs in the anaerobic digestion of EAS, including the variations of quantity and functional groups. The objective was to provide a basis for guiding the development of strategies to counteract the inhibitory effects of HSs. Ultimately, energy recovery from sludge via anaerobic digestion is expected to be enhanced.

2. Materials and methods

2.1. Sludge samples and inoculum preparation

Two kinds of EAS were studied in the present experiments, EAS from a lab-scale reactor fed with synthetic wastewater (S-EAS) and real EAS from a local WWTP (R-EAS). A lab-scale reactor (working volume 36 L) was operated in a sequencing batch mode (SBR) consisting of 0.5h wastewater filling, 20-h aeration, 2-h settling, 0.5-h effluent decanting, and 1-h idling. The reactor was equipped with a speed-adjusting stirring device (240 rpm) and heating device (25 °C). In order to rule out the interference of external HSs, the SBR was run with synthetic wastewater (Table S1) whereby the S-EAS discharged from the reactor was free of external HSs (Li et al., 2019b). R-EAS was taken from the secondary clarifier of a local WWTP (A²/O process with influent of 1 Mm³ d⁻¹) in Beijing. This WWTP mainly receives domestic wastewater with a sludge retention time of 22 days. S-EAS and R-EAS as substrates were fed respectively into two identical 4-L fermenters (Liu et al., 2016a) which later served as inoculum sources (termed as S-In from S-EAS fermenter and R-In from R-EAS reactor) for the following anaerobic batch tests.

2.2. Batch test for HSs dynamics monitoring

Batch tests were conducted to study the dynamics of the HSs in the digestion of two types of sludge with serum bottles as reactors. 17 serum bottles labeled as R1-R17 were used in the experiment. For R1-R2, 200 mL S–In, 100 mL S-EAS, and 3.6 g Hepes were prepared and cultured in the reactors while for R3-R17, 250 mL R–In, 150 mL S-EAS, and 4.7 g Hepes were filled into the bottles. The physicochemical characteristics of the two sludge samples are listed in Table S2. Hepes (Hao et al., 2017b) was added to maintain a neutral pH. Then, each bottle was flushed with N_2 before proceeding the anaerobic digestion at 35 °C and 150 rpm.

R1 and R2 were operated for 30 days while R3-R17 for 45 days. For Group 1 of S-EAS, HSs (HA and FA) contents were analyzed only before and after the digestion. After 30 days, the sludge in the two serum bottles was put together (600 mL) for analysis. For Group 2 of R-EAS, the HSs contents were analyzed in Day 9, 18, 27, 36, and 45 respectively. For each analysis, the sludge from three randomly selected serum bottles was mixed and analyzed.

2.3. HS extraction and analysis

The method recommended by the International Humic Substances Society (IHSS) was adopted to extract HA and FA (International Humic Substances Society, 1996). For R1-2, the total HA and FA contents in sludge samples were measured as total organic carbon (TOC) while for R3-17, the HA and FA content in liquid and solid phase were monitored separately. TOC was determined with a Shimadzu TOC-V CSH machine according to the standard methods (Liu et al., 2016b).

2.4. Oxygen-containing functional groups

The contents of carboxyl and phenolic hydroxyl groups in the structures of HA and FA were determined by potentiometric titration as follows. 100 mg extracted HA or FA was mixed with 150 mL ultra-pure water (Electrical Resistivity – $10 \text{ M}\Omega \text{ cm}$; Conductivity – $0.1 \,\mu\text{S} \text{ cm}^{-1}$) and the pH was then adjusted to 12 with 5 M NaOH to have the sample completely solubilized. Then, the pH of the solution was adjusted to 3 with HNO₃ which is the start point for titration. During the titration procedure, standardized NaOH (0.10 mol L^{-1}) was used as the titrat. The corresponding volumes of NaOH consumed at the point of pH = 8 and pH = 10 were recorded. The content of carboxyl groups was calculated according to the quantity of NaOH consumed to increase the pH of the solution from 3 to 8, and the content of phenolic hydroxyl group was calculated as the twice the amount of NaOH consumed to further raise from 8 to 10 (Ma et al., 2001).

2.5. Ultraviolet absorbance

Ultraviolet absorbance (UV) measurements of HAs and FAs were conducted from 800 nm to 200 nm using an Agilent Cary 5,000 UV–Vis–NIR spectrophotometer. Samples were added into the 0.05 M NaHCO₃ solution at a final concentration of 200 mg L⁻¹. Then, the solution was used to measure the absorption. SUVA₂₅₄ was obtained using UV–Vis absorbance at 254 nm, and then the values were normalized per mg TOC of the sample solutions (Weishaar et al., 2003). E₂/ E_3 refers to the UV–Vis absorbance ratio at 250 and 365 nm and E_4/E_6 represents the UV–Vis absorbance ratio at 465 and 665 nm.

2.6. FTIR spectra analysis

FTIR (Fourier transform infrared) spectra is a mainstream tool for determining functional groups and analyzing structural composition of HSs. 1 mg of freeze-dried sample and 100 mg of IR-grade KBr were mixed to get pellets, and then pellets were scanned using Thermo Fisher FTIR spectrometer from 4,000 to 400 cm^{-1} (Filip et al., 2000).

2.7. Two-dimensional correlation spectra

Two-dimensional correlation spectra (2D-CS) have the advantages of improving spectral resolution and reflecting the changing degree and order of different functional groups at different temperatures or over time. Correlation spectra in the region of $1,700-900 \text{ cm}^{-1}$ were processed with 2Dshige software from Kwansei-Gakuin University because the region of $1,700-900 \text{ cm}^{-1}$ contains many bands related to amides, carboxylic acids, aliphatic compounds and carbohydrates (Gamage et al., 2014). The interpretation of the graph was based on Noda's rule (Morita et al., 2001; Noda and Ozaki, 2005). Briefly, 2D-CS includes both synchronous and asynchronous plots. The synchronous plot contains both auto-peaks and cross-peaks, while the asynchronous plot only contains cross-peaks. The cross-peak in the red area is defined as the positive correlation intensities while the cross-peak in the blue area indicates a negative correlation.

3. Results

3.1. Variations of HS content in two reactors

Variation of HS content was assessed in samples from two digestion reactors run in parallel, one fed with HS-free sludge (S-EAS) and one with sludge obtained from full-scale wastewater treatment plant (R-EAS, Table 1). In the reactor fed with S-EAS, the concentrations of HA and FA before digestion were 370 ± 5 and $190 \pm 2 \text{ mg L}^{-1}$, respectively. After digestion, the concentrations of HA and FA increased a little bit to 390 ± 2 and $200 \pm 3 \text{ mg L}^{-1}$ respectively. Accordingly, the proportion of HSs in TS increased from 2.8% to 3.5% due to the

Table 1

Dy	namics	of HS	content	in	reactors	with	S-EAS	and	R-EAS	before	and	after	digestion	ı.
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Reactor		HA (mg L^{-1})	FA (mg L^{-1})	$HSs (mg L^{-1})$	The ratio of HSs in TS (%)
S-EAS	Before digestion After digestion	370 ± 5 390 ± 2	190 ± 2 200 ± 3	560 ± 13 590 ± 10	2.8 ± 0.12 3.5 ± 0.05
R-EAS	Before digestion After digestion	$1,162 \pm 3$ $1,100 \pm 13$	381 ± 6 343 ± 3	$1,543 \pm 10$ $1,443 \pm 40$	5.5 ± 0.64 NA ^a

^a NA-not available.



Fig. 1. Contents of HA and FA during digestion of R-EAS in liquid (a) and solid phase (b).

degradation of VSS. In contrast, the HS content in the sludge produced from real wastewater was significantly higher. The HS content in the R-EAS after digestion slightly decreased from $1,543 \pm 10 \text{ mg L}^{-1}$ to $1,443 \pm 40 \text{ mg L}^{-1}$ and both HA and FA decreased. Given the high HS content in the real wastewater, the HS content in R-EAS was substantially higher than in S-EAS and accounted for 5.5% of the TS.

The HA dominated the HSs and was one order of magnitude higher than FA both in liquid and solid phase at all times (Fig. 1). As digestion progressed, the content of HA and FA in the solid phase constantly decreased (Fig. 1b), which resulted in net decreases by 6.7% and 40.9% respectively. In the liquid phase, however, the content of HA and FA (Fig. 1a) gradually increased by 48.3% and 43.9% respectively.

3.2. Variations of oxygen-containing functional groups and humification degrees

Changes of carboxyl group, phenolic hydroxyl group, and total acidity in HA and FA before and after digestion of R-EAS are shown in Fig. 2. In both liquid and solid phase, the amount of carboxyl group and phenolic hydroxyl group in HA and FA increased after digestion (increased by $0.3-0.6 \text{ mmol g}^{-1}$ and $0.2-0.3 \text{ mmol g}^{-1}$, respectively), thereby leading to an increase of the total acidity (by $0.7-1.1 \text{ mmol g}^{-1}$) as well. Additionally, the content of oxygen-containing functional groups (carboxyl and phenolic hydroxyl groups) in

FAs was always a bit higher than in HAs, particularly in the liquid phase, which is consistent with a previous study (Kang et al., 2002). Regarding the comparison in the liquid and solid phase, the amount of oxygen-containing groups of HA and FA in the liquid phase was obviously higher than those in the solid phase before and after digestion (Fig. 2).

In addition, the aromatic and humification degree, molecular size, and condensation of HSs after digestion of R-EAS can also be interpreted by SUVA₂₅₄, E_2/E_3 , and E_4/E_6 indexes as listed in Table 2. SUVA₂₅₄ has a positive correlation with aromaticity of HSs while the indexes of E_2/E_3 and E_4/E_6 have a negative correlation with the humification degree, molecular size, and condensation (Peuravuori and Pihlaja, 1997; Polak et al., 2007). Obviously, the SUVA₂₅₄ for each HSs fraction (HA and FA in the liquid and solid phases respectively) increased after digestion of R-EAS while both E_2/E_3 and E_4/E_6 for all the parameters decreased after digestion. Compared with S-EAS (Table S3), the lower SUVA₂₅₄, and higher E_2/E_3 and E_4/E_6 of R-EAS before digestion indicated that the R-EAS had a lower degree of humification and aromaticity.

3.3. Structural analysis with FTIR spectra

For the HA fraction in the liquid phase, the intensity of the peaks at 2,925, 2,855, and 1,456 cm⁻¹ (assigned to aliphatic structures) was



Fig. 2. Contents of oxygen-containing functional groups in HA and FA before and after digestion of R-EAS.

Table 2

SUVA₂₅₄, E₂/E₃ and E₄/E₆ of HA and FA before and after digestion of R-EAS.

Subject	Before digestion				After digestion			
_	HA-L	FA-L	HA-S	FA-S	HA-L	FA-L	HA-S	FA-S
SUVA ₂₅₄ (L·(mg·m) ⁻ 1)	0.98	1.22	2.47	1.77	2.79	3.08	5.85	5.20
$\begin{array}{c} E_2/E_3\\ E_4/E_6\end{array}$	6.41 13.48	5.81 12.37	4.59 11.50	5.61 12.13	3.63 8.51	3.30 8.01	2.18 7.11	3.10 7.79

increasing gradually until the 27th day and after that, the peak intensity weakened (Fig. 3a). Besides, the peak at $1,050 \text{ cm}^{-1}$ (polysaccharideslike substances) decreased, which was also attributed to oxidation. No obvious changes of the characteristic peaks on the FTIR spectra of HA in the solid phase (Fig. 3b) occurred during digestion, which reflects the refractory nature of HA in the solid phase. However, the absorption peak at $1,655 \text{ cm}^{-1}$ (belonging to aromatic C=C and C=O of amide I) finally became prominent in the spectra of both in the liquid and solid phase.

In terms of FA, the structure change presented some distinct features compared with HA (Fig. 3c and d). For FA in the liquid phase, the absorption peaks at 2,925 and 2,855 cm⁻¹ almost disappeared as digestion progressed and some other absorption peaks also became less visible. The absorption peak at 1,655 cm⁻¹ assigned to aromatic compounds was prominent at the end. The FA in the solid phase showed some similar structural dynamics to the liquid phase and the aromatic moieties (1650 cm⁻¹) also dominated after digestion.

3.4. Analysis of two-dimensional FTIR correlation spectra

The key interpretation of the 2D-CS graph was presented in Fig. S1 and summarized in Table S4. In the sp row in Fig. S1, the red intensity in the diagonal represents the degree of change. For HA in the liquid and solid phase (sp row in Figs. S1a and S1b), the amide group

 $(1,655 \,\mathrm{cm}^{-1})$ changed the most, followed by polysaccharides $(1,550 \,\mathrm{cm}^{-1})$ and then the carboxyl group $(1,050 \,\mathrm{cm}^{-1})$. For FA in the liquid phase and solid phase, polysaccharide $(1,550 \,\mathrm{cm}^{-1})$ and aliphatic groups $(1450 \,\mathrm{cm}^{-1})$ changed most significantly while the carboxyl group was little affected. The results imply that some more reactive functional groups like aliphatic group were firstly and quickly oxidized.

In addition, the changing order (cross-peaks in the ap row of Fig. S1 and Table S4) may be more meaningful to indicate the sequences of attack of various functional groups. For HA and FA in liquid or solid phase, the aliphatic group $(1,450-1,456 \text{ cm}^{-1})$ was always the first to be modified while the polysaccharide group $(1,000-1,055 \text{ cm}^{-1})$ was almost the last one to be degraded. Amide and carboxyl groups were in the middle of the sequence.

The changing direction of each functional group (increase or decrease) can also be gained from the correlation index (Table S4). For the HA in the liquid phase, the intensity of amide II and III, carboxylic acids, polysaccharides-like group, and aliphatic group decreased during digestion while in the solid phase the intensity of amide I and II, aromatic ring skeleton and carboxylic acids in the structural composition of HA-S increased. Similarly, for the FA in the liquid phase, the intensity of amide II and carboxylic acids deceased and in the solid phase, amide II and aliphatic group decreased while polysaccharides-like group accumulated. Obviously, FA had a higher humification degree due to the consumption of aliphatic groups.

4. Discussions

4.1. Content dynamics of HSs in AD

In general, the HSs in the sludge derive from the degradation and polymerization of plants, animal residues, and dead microorganisms, while lignin is the primary precursor of HSs formation. It is still not clear whether in anaerobic conditions HS can be removed by biotic or abiotic processes or whether HS can be formed. The reactor fed with S-EAS was designed to shield the influence of external HSs and to observe whether HSs can be formed *in situ*. However, the HS content at the beginning of the operation was unexpected at 560 mg L⁻¹. Although the bulk concentration of HSs and the mass ratio in the reactors fed with S-EAS increased slightly, the absolute content of HSs remained almost unchanged by considering the reduction of volume and VSS. However, the reduction of HS content in the reactors fed with R-EAS was more pronounced, suggesting that the HSs in reactors fed with S-EAS and R-EAS presented some differences in properties.

Based on the source of S-EAS and R-EAS, this difference might be explained from two aspects. One probably is the different humification degree of the HSs in S-EAS and R-EAS. This can be verified by the higher humification degree and aromaticity of S-EAS compared with R-EAS (E_2/E_3 in Table S3 and Table 2). As mentioned in the properties of sludge used in the work, S-EAS came from a reactor operated under a persistent and longer aerobic condition Where the mineralization of HSs was more significant (Li et al., 2017; Tang et al., 2018). Thus, when the S-EAS were transferred to AD, they would be hardly decreased further. From the R-EAS point of view, the compositions of HSs in real sludge is more complex and with a lower degree of humification as reflected by the SUVA₂₅₄ and E_4/E_6 (Table 2 and S4) (Li et al., 2013). Thus, there could be a higher proportion of moieties in HSs with small molecular weight and high content of aliphatic moieties which can be relatively readily oxidized (Li et al., 2017). Moreover, the 2D-CS result verified that the aliphatic moieties of HSs were the first to change in the digestion process. The oxidation of aliphatic moieties probably contributed to the decrease of HSs content in the reactor with R-EAS. Particularly under a low organic loading rate (0.06 mg VSS (mg VSS·L)⁻¹ in the reactor with R-EAS over 0.077 mg VSS·(mg VSS·L)⁻¹ in the reactor with S-EAS, the aliphatic moieties of HSs was more readily oxidized (Lipczynska-Kochany, 2018; Provenzano et al., 2016). Thus,



Fig. 3. FTIR spectra comparison along the digestion of R-EAS: HA in liquid (a); HA in solid (b); FA in liquid (c); FA in solid (d).

different organic loading rates applied in two tests might be another reason accounting for the difference.

In addition, although the formation of HSs under anaerobic condition was not observed in the present study, the unexpected presence of HS in the reactors fed with S-EAS demonstrated the probable formation of HSs during the culture of S-EAS in the SBR (Stevenson, 1994; Lipczynska-Kochany, 2018). Several previous studies also reported the evidence of bio-synthesis of HSs (Li et al., 2017). When sodium acetate was used as the sole carbon source in the influent to an SBR system, HSs were found in the EPS of sludge (Guo et al., 2012). In fact, HSs were not only one of the important components of extracellular polymeric substances (EPS), but also appeared in the cells (Li et al., 2013). In addition, the unexpected HSs also probably came from fermenters where the inoculum was cultured. It is worth noting that the variation of the HSs during AD was very small relative to the total amount of HSs in the reactor. Thus, the quantity variation of the HSs is not likely to impose a significant impact on digestion performance, but the dynamics and distribution of HSs in the solid and liquid phase may influence the inhibition pattern which is discussed below.

4.2. Structure dynamics of HSs in AD

In terms of the structural dynamics of HSs in AD, both HA and FA were getting relatively a higher humification degree as indicated by the increase of the total acidity (carboxyl and phenolic moieties). Moreover, the digestion led to a significant reduction of the aliphatic moieties and an increase of aromaticity of HSs in the liquid and solid phase, especially the FA. From the structural point of view, the aliphatic moieties of HSs are linear, simple, and more vulnerable compared with the aromatic part. Thus, upon exposing HS to the anaerobic digestion environment, the aliphatic moieties would be more easily oxidized, as the peaks denoting aliphatic structures weakened or disappeared (Fig. 3), with concomitant enrichment of aromatic moieties (Shirshova et al., 2006). Thus, anaerobic digestion makes HS more aromatic and condensed.

It is worth noting that there was a trend of HS release from the solid phase into the liquid phase in the reactors with R-EAS. It means that the anaerobic digestion promoted the solubilization of HSs slightly. This phenomenon was also reported in the previous study and explained by the desorption and release of HSs from sludge particles (Qi et al., 2004). The results from the present study, particularly the dynamics of oxygenfunctional groups, could provide justification for this solubilization. The increase of oxygen-containing groups after digestion would enhance the hydrophilicity of HSs and increase their solubility, thus facilitating their transfer into the liquid phase which is consistent with the higher amount of oxygen-containing groups detected in HSs from the liquid phase (Fig. 2).

According to the previous research, HSs could have a positive or negative impact on each stage of anaerobic digestion of sludge (Lipczynska-Kochany, 2018). As summarized in Table 3, the impact of HA on hydrolysis was not always negative. The surfactant-like HA could probably form protective micelles or facilitate the enzyme-substrate

Table 3

Summary of the inhibition impact and pathways from HSs.

Stage	Hydrolysis	Acidogenesis	Methanogenesis	Note
Li et al. (2019b)	Negative; deactivating the enzymes	Positive ; stimulating the enzyme or being electron acceptor or shuttle	Negative; deactivating the enzymes	Commercial HA (lower aromaticity)
Liu et al. (2015)	Positive ; promoting the contact of sludge and enzymes	Positive; being an electron acceptor	Negative ; blocking the way of acetyl- CoA to 5-methyl-THMPT	Commercial HA (higher aromaticity, greater hydrophobicity)
Zhou et al. (2014)	na	na	Positive ; promoting <i>Geobacter</i> spp. growth and being an electron acceptor	Humic surrogate compound
Hartung (1992)	Positive; stimulating the activity	of microorganisms and metal regulation	n	HA from peat
	Neutral;			HA from soil
Azman et al. (2017)	Negative ; deactivating the enzymes	na	na	Commercial HA

na – not available.

interaction to promote hydrolysis. The solubilization of HSs might account for this discrepancy. On the one hand, the soluble HSs could be the more active inhibitor to the hydrolysis because of their high mobility and easy access to enzymes (Li et al., 2019b). On the other hand, the increased amount of oxygen-containing group of HSs could also promote the solubilization of other organic compounds by disassociation from HSs as well as the hydrolysis. In addition, HA extracted from different sources (peat or soil) also presented different impacts on hydrolysis. In general, HSs have been implicated in hydrolysis inhibition due to enzyme deactivation. These contradictory results imply that the species, structure, and functional groups of HSs jointly determine their impact on each stage of AD. Thus, the dynamics of HSs in anaerobic digestion can help propose the corresponding mitigation strategies.

4.3. Guidance to alleviate the possible inhibition from HSs

So far, limited research has been reported on HSs in liquid and solid phases separately as in the present study. It is interesting to note that the soluble HSs was not very high compared with the total HSs, especially the HA of which the concentration was always lower than 50 mg L^{-1} . In studies focusing on the inhibitory impact of HA on hydrolysis, the HA was reported to pose inhibition at the dosage of $0.5-8 \text{ g L}^{-1}$ (Brons et al., 1985; Fernandes et al., 2015; Azman et al., 2017). This variation may be attributed to the fact that the actual amount of HSs posing inhibition was different from the amount of artificially dosed. Thus, the pattern of hydrolysis inhibition after addition of HS is of no relevance to the present study, where the concentration of soluble HS was determined by the rate of their dissolution from the solid phase. Thus, any applied methods to reverse the inhibitory effect of HSs should consider the distribution of HSs in liquid and solid phase.

In addition, the results here demonstrated that the structure of HSs in ADs is changing slightly and dynamically. This could partly explain why the previous studies recommended various amounts of inorganic cation addition to the mitigation of HSs' impact (Azman et al., 2015, 2017). In the experiment from Azman et al. (2015), pulse addition of CaCl₂ at short HRTs (2–4 h) was applied while in the study of Azman et al. (2017) the CaCl₂ was semi-continuously dosed. Obviously, it was the addition strategy that led the different results with the same amount of cation addition. By dosing CaCl₂ more frequently, the cations can shelter the adverse impact of HSs dynamically generated immediately. Moreover, the free cations could also enhance the microbial activity by leading more enzyme production (Azman et al., 2015).

As summarized in Table 3, enzyme deactivation by HSs is one of the main reasons compromising the performance of AD (Azman et al., 2017). The present study showed that the HS content in the solid phase was higher. This part of HSs is usually associated with and adsorbed on the surface of biomass or polymers and might prevent the enzymes from accessing the substrate. As such, the dosage of inorganic cations is effective to free the enzymes from the HSs but can do little if the HSs deposited on the surface of the substrate. Moreover, the trap of substrate because of the HSs and the dosed cations may increase the access

difficulty of enzymes to substrates. Under this circumstance, pretreatment strategies (mechanical, physical, or chemical methods) can be more feasible to reverse the inhibitory impacts.

5. Conclusions

The dynamics of HSs (including content, structure, and oxygencontaining functional groups) in anaerobic digestion of sludge grown on synthetic and real wastewater was studied in the present work. A transformation among quantity in liquid or solid phase, structure, and functional groups occurred during the anaerobic digestion. According to the above results, some key conclusions can be summarized here.

- 1) With real sludge, the total HSs content declined slightly by 6.5%. The contents of HA and FA in the liquid phase increased by 48.3% and 43.9% respectively after digestion; on the other hand, the contents of HA and FA in the solid phase declined by 6.7% and 40.9% respectively.
- 2) The changes of HA and FA during digestion of real sludge was caused by losing aliphatic moieties and enriching aromatic moieties in their structural compositions, which finally increased the degrees of humification, aromaticity, and the amount of oxygen-containing functional groups.
- 3) The FA content in the real sludge is drastically lower than HA and the FA is more easily to be humified than the HA due to the simple structure and low molecular size.
- 4) Strategies to alleviate the inhibition effect of HSs should consider the HSs in different phases, solid and liquid phase, as HSs in different phases presented different inhibition degree.

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Appendix A. Supplementary data

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