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**Transformation of dissolved organic matters produced from
alkaline-ultrasonic sludge pretreatment in anaerobic digestion:
from macro to micro**

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Abstract

Soluble organic compounds released by alkaline (ALK), ultrasonic (ULS) and combined alkaline-ultrasonic (ALK-ULS) pretreatment as well as their transformation in the anaerobic digestion systems were investigated. The maximum methane production of 197.1 ± 3.0 mL CH₄/g tCOD_{feed} was observed with ALK-ULS pretreated sludge (pH 12 and specific energy input of 24 kJ/g TS). The combined treatment likely enhanced the sludge solubilization and produced more low molecular weight (LMW) substances, which were beneficial to improve the biogas generation rate. However, such pretreatment released not only easily biodegradable substances but also more recalcitrants, such as humic substances (HS) and complex high molecular weight (HMW) proteins. Thus, more residual dissolved organic matters (DOMs) were detected after digestion, which may pose adverse effects on the downstream water treatment. Refractory HS and hydrophobic dissolved organic carbon (HO DOC) were the main components of the residual DOMs, which accounted up to 35.0% and 22.3% respectively. At the molecular level, a large amount of residual polycyclic steroid-like matters, alkanes and aromatics were identified. Specific higher MW residual compounds, e.g. polar metabolites (like dipeptide, benzene and substituted derivatives), and non-polar lipids (like diacylglycerols, long chain fatty acids, alkenes, flavonoids, sphingolipids, glycerolipids, glycerophospholipids and their derivatives) were also identified. The results indicate that further polishing steps should be considered to remove the remaining soluble recalcitrant compounds. This study helps to understand the insight of sludge treatment from macro to micro level.

Keywords: Pretreatment; Anaerobic Digestion; Alkaline; Ultrasonic; Dissolved organic matters; Recalcitrant Compounds

1. Introduction

Anaerobic digestion (AD) is widely used to recover bioenergy, reduce sludge volume and stabilize the biomass (Yuan and Zhu 2016). However, sludge hydrolysis, which is considered as the rate-limiting step in digestion, requires further development in both research and application (Carrere et al. 2010, Liu et al. 2016). Many sludge pretreatment approaches, such as thermal, chemical, mechanical and biological pretreatment have been developed to accelerate the hydrolysis step and improve the subsequent AD efficiency (Abelleira-Pereira et al. 2015, Neumann et al. 2017, Wei et al. 2017).

Among above methods, alkaline (ALK) pretreatment has been widely applied as it is the most effective chemical method, and can greatly solubilize particulate matters and enhance the sludge digestibility in a simple setup (Ruiz-Hernando et al. 2014, Yang et al. 2013). Ultrasonic (ULS) pretreatment also receives attention and has been implemented in many full-scale plants (Zhang et al. 2007). Nevertheless, high energy consumption limits the broad application of ultrasonication (Zhang et al. 2008). By combining with other pretreatment methods, the energy requirement of ULS can be reduced, and the sludge disintegration as well as biogas production can be improved too (Zawieja et al. 2008). For instance, the synergistic effect of combined ALK-ULS pretreatment has been reported recently (Kim et al. 2010, Li et al. 2015).

Generally, the methane production performance is closely related to sludge solubilization efficiency (Carrere et al. 2008). However, most of the research to date mainly focused on the relationship between the total organics removal (volatile solids removal in particular) and the methane production with/without sludge pretreatment. The detailed correlation between the role of specific organics and biogas generation has not been systematically investigated.

In AD process, dissolved organic matters (DOMs) are considered as the direct assimilable carbon source to microorganisms compared with particulate organic matters. It is not clear how these DOMs are produced in the pretreatment step and how the key DOMs are transformed during subsequent AD process. Besides, the information on the potential inhibitory compounds produced during pre-treatment as well as the recalcitrant compounds remained after anaerobic digestion is still lacking. Such study is particularly important when a plant has a water recycling requirement.

The main objectives of this study are to (1) explore the transformation of DOMs after ALK and/or ULS pretreatment, (2) study the impact of such organics on the subsequent AD process, and (3) identify the recalcitrant DOMs after AD process. The solubilization and biodegradability of WAS with single or combined ALK-ULS pretreatment were compared and evaluated. This study, for the first time, provided detailed insights into the transformation of soluble organic matters in sludge treatment process at the molecular level. In addition, some refractory compounds that may be potentially formed during pretreatment or AD were also identified and discussed.

2. Material and methods

2.1 Sludge source

The feed sludge (pH 6.7) was waste activated sludge (WAS) collected from a local wastewater treatment plant in Singapore. It was settled at 4 °C for 24 h before use. The anaerobic sludge collected from a mesophilic anaerobic digester in the same plant was used as the inoculum. The characteristics of WAS and seed sludge are listed in Table 1.

2.2 Reagent and chemicals

Chloroform, dichloromethane, acetone and n-hexane of GC-MS grade were purchased from Merck KGaA (Germany). Acetonitrile, isopropanol, water and methanol (all LC-MS grade) were purchased from Sigma-Aldrich (United States). Formic acid

and ammonium formate were purchased from Sigma-Aldrich. The alkane standard mixture (C10 – C40, 50 mg/L) was purchased from Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q water process (Millipore Advantage A10, Merck, France).

2.3 Sludge pretreatment

2.3.1 ALK pretreatment

The pH value of WAS was adjusted from 6.7 to 9, 10, 11 and 12 respectively using NaOH solution (5 mol/L). After that, the WAS samples were incubated for 30 minutes at room temperature, then neutralized to pH 6.7 using HCl solution (5 mol/L) (Jin et al. 2009). Deionized water was used to top up the final sample to the same volume so that the solids concentration in all the samples can be maintained comparable. Triplicates were prepared for each type of pH sludge.

2.3.2 ULS pretreatment

The ULS treatment was operated using an ultrasonicator (Q700, QSonica, Newtown, CT, USA) with the frequency of 20 kHz. The tip of probe was placed 1 cm into sludge samples. The temperature of sludge during ultrasonic pretreatment was controlled within 30 ± 5 °C by the ice bath. The specific energy input (SEI) for sludge samples was set at 0, 6, 12, 18 and 24 kJ/g VS respectively. Triplicates were prepared for each ULS test.

2.3.3 ALK-ULS pretreatment

It was reported that the pretreatment performance was much better with the sequence of ALK-ULS than ULS-ALK (Tyagi and Lo 2012). Thus, feed sludge samples were firstly pretreated with alkali at the pH values of 6.7, 9, 10, 11 and 12 respectively as described in Section 2.3.1, and then neutralized to pH 6.7. The ALK treated samples

were further treated with SEI of 0, 6, 12, 18 and 24 kJ/g VS as described in Section 2.3.2. Triplicates were prepared for each type of sludge.

After pretreatment, all the treated sludge samples were split into two groups. One group was used for measurement of TS, VS, TSS, VSS, total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), disintegration degree (DD), PN, PS analyses and EPS extraction. The other group was used as feed for anaerobic digestion.

Disintegration degree (DD) was calculated with Eq. (1), which can directly show the degree of particulate substance solubilized by pretreatment (Jin et al. 2009).

$$DD\% = \frac{sCOD_p - sCOD_0}{tCOD_0 - sCOD_0} \times 100 \quad (1)$$

where $sCOD_p$ is the sCOD of pretreated sludge, $sCOD_0$ is the sCOD of untreated sludge, and $tCOD_0$ is the tCOD of untreated sludge.

2.4 Anaerobic digestion process

The anaerobic digestion experiment was carried out using an automatic methane potential test system (AMPTS II, Bioprocess Control Company, Sweden). Each reactor had working volume of 400 mL and headspace of 150 mL. The inoculum to substrate VS ratio was 0.98. Reactors were purged with N_2 to remove dissolved gas. All experiment was conducted at 35 °C for 30 days with semi-continuous stirring (60 s on and 10 s off, 80 rpm). Control experiment was operated with raw WAS as feed sludge. Blank tests (inoculum and deionized water) were carried out in duplicates to provide the background biogas production from the inoculum. The blank methane production was subtracted. Four replicates were carried out for each type of pretreated sludge. Among them, two replicates were continuously operated for 30 days and samples were taken on the last day for the analysis of residues. The other two replicates were sampled at 0, 2nd, 5th, 10th, 16th and 30th day during AD to monitor the changes of DOMs.

2.5 Analytical methods

2.5.1 Characterization of feed sludge and anaerobic sludge

The measurement of TS, VS, TSS, VSS, sCOD and tCOD was based on Standard Methods (APHA, 2005). pH was measured with a pH meter (Mettler-Toledo, model S220). For the measurement of soluble substance, sludge samples were centrifuged at 10000 rpm for 10 min. Then the supernatant was collected and filtered through 0.45 μ m nylon membrane filters.

2.5.2 Extracellular polymeric substances (EPS) extraction and analysis

EPS of pretreated sludge samples was extracted following the protocols described in the literature (Xiao et al. 2017) and collected as soluble EPS (SB EPS), loosely bound EPS (LB EPS) and tightly bound EPS (TB EPS) separately. The extracted supernatant was filtered through 0.45 μ m nylon membrane filters for the analysis of dissolved organic carbon (DOC), proteins (PN), polysaccharides (PS) and humic substance (HS). Soluble PN was determined using the protein assay kit (ThermoFisher, USA) by the modified Lowry-Folin method. Soluble PS was measured by the phenol-sulfuric acid method (Dubois et al. 1956). DOC was determined using a total organic carbon (TOC) analyzer (Shimadzu, Japan).

2.5.3 Size exclusion chromatography (SEC) coupled with organic carbon detection and organic nitrogen detection (LC-OCD-OND) analysis

To quantify the major DOMs with different molecular weight and chemical properties, SEC associated with LC-OCD-OND system (DOC-LABOR, Karlsruhe, Germany) was employed. The detailed methods can be found in Xiao et al. (2016).

2.5.4 Solid phase extraction (SPE) and liquid-liquid extraction (LLE) for Gas Chromatography–Mass Spectrometry (GC-MS)

Pretreatment step to extract organic compounds was carried out with SPE cartridges (Waters Oasis HLB, Waters Corporation, United States). Then the filtrate obtained through SPE cartridge was treated by LLE. The operational procedures of SPE and LLE can be found in Kunacheva et al. (2017).

2.5.5 GC–MS

Samples prepared from LLE and SPE were analyzed using GC-MS (GCMS-QP2010ULTRA, Shimadzu) separately. Description and analysis of GC-MS operation process can be found in Kunacheva et al. (2017). The chromatographic peaks were identified by both analysis of the mass spectrum, retention time and comparison with the NIST11 library (National Institute of Standards and Technology, Gaithersburg, MD, USA, <http://www.nist.gov/srd/mslist.htm>). Each compound was semi-quantified using the alkane with the closest retention time (Table S4).

2.5.6 Metabolites and lipids extraction and analysis

The metabolites fraction and lipids fraction were extracted from the lyophilized substances following the procedures in Tiphara et al. (2017), and analyzed by ACQUITY UPLC (Waters, USA) coupled with a Xevo G2-XS QToF mass spectrometer (Waters, U.K.) with an electrospray ionization (ESI) source. Detailed description and analysis of Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC-MS) operation process can be found in Tiphara et al. (2017). EZinfo (version 3.0.3.0, Umetrics) was used for multivariate analysis of compounds in raw WAS, ALK-ULS pretreated sludge and digested sludge samples respectively. The most significant components were determined by orthogonal projection to latent

structures discriminant analysis (OPLS-DA) and then exported from S-plot back to Progenesis QI for further analysis and identification. Polar and lipid biomolecules were identified using (1) MetaScope by searching compounds against structural databases (SDFs) for *E. coli* (<http://ecmdb.ca/>), LIPID MAPS (<http://www.lipidmaps.org/>), HMDB (<http://www.hmdb.ca/>), and ChEBI (<https://www.ebi.ac.uk/chebi/>) and (2) Chemspider by searching compounds against the KEGG database (<http://www.genome.jp/kegg/>).

2.6 Kinetics analysis

Methane generation data was fitted to a First-Order Model using Eq (2) to estimate the hydrolysis rate coefficient (k) and biochemical methane potential B_0 (Jensen et al. 2011).

$$B(t) = B_0 (1 - e^{-kt}) \quad (2)$$

where $B(t)$ is the biochemical methane yield at time t , B_0 is the biochemical methane potential, and t is time.

2.7 Statistical analysis

Pearson's correlation was applied to study the linear correlation between biogas production, operation parameters and soluble organics by the software SPSS version 19.0 (Niu et al. 2013). The extent of correlation was evaluated by the Pearson's correlation coefficient (R), which can range between -1 and +1, where -1 denotes a perfect negative correlation, +1 indicates a perfect positive correlation, and 0 denotes no relationship. A two-tailed t-test for the null hypothesis that regression slope is zero was carried out to determine p value. The correlation will be considered as statistically significant with probability (p value) less than 0.05.

3. Results and discussion

3.1 Effects of ALK and/or ULS pretreatment on sludge properties

3.1.1 Effects on EPS distribution and compositions

The effects of ALK (pH 6.7 - 12) or ULS (0 - 24 kJ/g TS) as well as ALK-ULS pretreatment on the sludge EPS fractions are present in Figure 1. For raw sludge, TB EPS fraction was the most dominant in EPS matrix. After pretreatment, part of EPS and microbial fragments would be solubilized and shifted from the inner layers (TB EPS and LB EPS) to outer layers (LB EPS and SB EPS), leading to the increase of SB EPS and LB EPS fractions. It is worthy to note that DOMs were more dominant in SB EPS after pretreatment. This part of DOMs is in fact more accessible to anaerobic microorganisms. This confirms that pretreatment can improve the accessibility of organic matters in sludge fractions.

Clearly, both ALK or ULS pretreatment could improve the sludge solubilization with the increased alkali dosage or energy input (Figure 1). The optimal solubilization for individual ALK and ULS pretreatment was at pH of 12 and SEI of 24 kJ/g TS respectively. Besides, when ultrasonication was applied to the ALK pretreated sludge, the remarkable increase of solubilization extent was observed. It is likely that cell walls weakened by ALK were easily destructed by ULK, resulting in the direct release of the soluble intracellular organics. Therefore, the combined pretreatment under conditions of pH 12 and SEI 24 kJ/g TS showed the best solubilization performance of 247.04 mg DOC/g VS. In order to investigate the transformation of DOMs with different pretreatment methods, the following three optimal conditions were chosen, namely, combined pH 12 and SEI 24 kJ/g VS (ALK12-ULS24), individual ALK pretreatment at pH 12 (ALK12), ULS pretreatment at SEI 24 kJ/g VS (ULS24).

3.1.2 Effects of ALK and/or ULS pretreatment on DOMs compositions

DOMs at the different molecular weight of the pretreated sludge were analyzed by LC-OCD-OND with more specific fractions. As described in Figure 2, the observed DOMs were mainly divided into HO DOC (hydrophobic dissolved organic carbon), biopolymers (> 20 kDa, including HMW PS, HMW PN and amino-sugars), humic substances (HS), building blocks (300 - 500 Da), LMW neutrals (< 350 Da, including alcohols, aldehydes, ketones and mono-oligosaccharides) and LMW acids (< 350 Da) in terms of respective properties and molecular weight. HO DOC was calculated by subtracting HI DOC (hydrophilic dissolved organic carbon) from the total DOC concentration (Huber et al. 2011).

The concentration of HI DOC in ALK12-ULS24 pretreated sludge was clearly higher than that in other pretreated sludge as well as in control samples (Figure 2). The concentration of HMW PN was higher than HMW PS regardless of pretreatment methods, which is reasonable as 40 - 50% of dry cell weight is PN (Wei et al. 2014). Besides, the proportion of HMW PN in biopolymers increased from 60% in untreated sludge to 73.5%, 82% and 80% in ALK12, ULS24 and ALK12-ULS24 pretreated sludge respectively. It implies that ULS pretreatment may release more HMW PN than ALK pretreatment did. Conversely, ALK pretreatment likely released more HMW PS, as the biopolymers composed of HMW PN and HMW PS.

Although the concentration of HMW PN and HMW PS did not increase much with ALK12-ULS24 treatment compared to ULS24 treatment alone, the total PN and PS increased greatly (Figure S2a, Figure S2b), implying the release of LMW PN and LMW PS was enhanced by ALK treatment. Thus, the main reason for the enhanced DOMs release from combined ALK-ULS pretreatment is likely due to the increase in LMW substances.

Nevertheless, ALK treatment also released a high concentration of HS (52.87 mg/L -C). The concentration of building blocks (68.89 mg/L -C), which are the breakdown products of HS, was found much higher with combined treatment (Figure 2). Thus, ALK12-ULS24 treatment resulted in large amount of HS-like matters, including the complex HS and building blocks. Previous findings reported that part of HS belonged to hydrophobic substances (Penru et al. 2013). In this study, the concentration of HO DOC was lower with ALK treatment, indicating that ALK treatment may be in favour of generating hydrophilic HS rather than hydrophobic HS.

Statistical analysis demonstrates that strong or moderate correlation was found between SEI with HI DOC, HMW PN, HMW PS, LMW neutrals (Table 2) and the correlation was significant ($p < 0.01$), which suggests that the ULS treatment was more likely to be a physical way to release these organics that were originally embedded in sludge flocs rather than through chemical reactions. Meanwhile, a moderate correlation ($R = 0.616$, $p < 0.01$) was observed between pH and LMW acids, indicating the formation of LMW acids may be partly due to the hydrolysis of organic matters by the reaction of alkali. Additionally, sludge DD had a strong positive correlation with SEI ($R = 0.792$, $p < 0.01$) but a weak correlation with pH ($R = 0.466$, $p < 0.05$), which is in agreement with the reports that ALK treatment at high pH levels played more important roles than that at low pH values in increasing sludge disintegration prior to subsequent ULS pretreatment (Kim et al. 2010). It further confirms the different roles and mechanisms of ULS and ALK treatment in sludge disintegration. The strong correlation ($R = 0.792$) of statistical significance ($p < 0.01$) between DD and SEI suggests ULS treatment is more controllable in application.

3.2 Effects of pretreatment on methane production

The biodegradability of different pretreated sludge is shown in Figure 3. The cumulative methane production (CMP) increased rapidly at the beginning and then gradually reached a steady state during AD process (Figure 3B). This confirms that all pretreatment could promote the total biogas production compared to control systems. However, the individual ALK and ULS treatment behaved differently in facilitating the methane production, with the higher initial methane generation rate from ULS treatment (Figure 3A). In this study, the methane production rate by ULS pretreatment is higher than other reported studies (Ruiz-Hernando et al. 2014), in which the similar SEI but higher TS content were used. Such performance difference may be related to different sludge characteristics, e.g. sludge concentrations.

ALK12 pretreatment increased methane production by 11.8% (166.0 ± 5.0 mL $\text{CH}_4/\text{g tCOD}_{\text{feed}}$), while the methane production of ULS24 treatment increased by 20.0% (178.2 ± 3.1 mL $\text{CH}_4/\text{g tCOD}_{\text{feed}}$) (Figure 3). To compare, combined ALK12-ULS24 increased the methane yield by 32.8% (197.1 ± 3.0 mL $\text{CH}_4/\text{g tCOD}_{\text{feed}}$). It has been reported that inhibitory effect from Na^+ may affect the methanogens (McCarty and McKinney 1961), hence, the optimum pretreatment conditions for maximum solubilization may not be the best conditions for maximum biogas production. In this study, Na^+ concentration at the highest ALK dosage (0.018 mol/L) is far lower than the inhibition threshold (> 0.2 mol/L) (Hierholtzer and Akunna 2012). It was found out that the optimal pretreatment condition (ALK12-ULS24) for maximum solubilization was also the best condition for maximum biogas production. If the CMP from control reactors is used as the baseline (148.4 ± 2.1 mL $\text{CH}_4/\text{g tCOD}_{\text{feed}}$ till day 30), ALK12, ULS24 and ALK12-ULS24 groups could achieve the baseline value on day 14, 6 and 7, respectively. These findings are of great significance in reducing the system solids retention time (SRT) and reactor size. It should be noted that the hydrolysis rate

coefficient of organics in ALK12 treated reactors ($k = 0.222 \text{ day}^{-1}$) was lower than that in control reactors ($k = 0.232 \text{ day}^{-1}$), whereas the hydrolysis rate coefficient in ULS24 treated reactors ($k = 0.277 \text{ day}^{-1}$) was higher than that in control (Table S2). The results suggest that ULS24 caused an increase in hydrolysis coefficient, but ALK12 did not. The increase in hydrolysis coefficient for ULS24 is relatively small. The main effect of pretreatment is therefore an increase in bioavailability rather than in the speed of degradation. From Figure 3A, ALK treated sludge had a slower initial methane production rate compared to the ULS pretreatment. Detailed explanation will be presented below.

3.3 Transformation of DOMs during AD

To study the transformation of DOMs, samples were withdrawn from control and ALK12-ULS24 sludge digesters on 0, 2nd, 5th, 10th, 16th, 30th day. The characterization and comparison of DOMs in the two digesters are presented in Figure 4. In control digesters, the initial soluble substances concentration was very low (Figure 4), and the concentration was not significantly changed during the following 30 days digestion regardless of the biogas production rate. It seems the availability/accumulation of those compounds was somehow limited by upstream bioreactions. Hydrolysis step is known to limit the overall reaction rate in anaerobic digestion (Carrere et al. 2010), and the subsequent bioreaction kinetics can be much faster. The relatively stable and low concentration of soluble substances observed during AD may confirm hydrolysis was the limiting factor when there was no pretreatment performed. HMW DOMs, including HMW PN and HMW PS, were digested gradually to a lower concentration (Figure 4). On the contrast, some compounds, such as LMW neutrals and building blocks even increased in the first 10 days, which may due to the hydrolysis of biopolymers, HS or some particulate substances (Figure 4).

Different from the control reactors, a large amount of DOMs were available for AD after pretreatment. The majority of them, such as HO DOC, HMW PN, HMW PS, building blocks and LMW neutrals, were reduced rapidly within the first 5 days. The results showed good agreement with the trend of methane production (Figure 3). During AD process, part of biopolymers was hydrolyzed or degraded into LMW neutrals and LMW acids, leading to the elevation of some organics. The amount of HO DOC increased more than 2-fold within the first 2 days and then reduced gradually during digestion. This phenomenon indicates that HO DOC generated by anaerobic bacteria seemed easier to be degraded than that produced by pretreatment step. It is also likely that HO DOC-degrading bacteria needs some acclimation period at the early stage of AD. Generally, LMW DOMs was considered more easily biodegraded than HMW DOMs (Li et al. 2016). These suggest HO DOC generated by bacteria may consist of LMW alkanes and lipids, while those produced by pretreatment were more likely to be hydrophobic HS and HMW hydrocarbons. LMW acids also increased significantly at the beginning, which was mostly associated with the acidification of other organic matters.

PN and PS are the main components in WAS (Wilén et al. 2003). The anaerobic transformation of the two is very important in sludge reduction and energy recovery. It was found that the consumption of HMW PS was slower than HMW PN (Figure 4). It suggests HMW PS was probably more rate-limiting than HMW PN in hydrolysis step. This agrees that the majority of PS exists in the cell walls, which is resistant to the hydrolysis (Li and Noike 1992). From the previous result in Section 3.1.2, ALK12 and ALK12-ULS24 pretreatment likely released a higher percentage of HMW PS in comparison with ULS24 treatment. Thus, this could be one of the reasons for the slower initial methane generation rate in ALK-related pretreated sludge reactors.

In addition, HS was not consumed but accumulated during the first 10 days in both reactors, then declined slightly during the latter period. This proves that HS was less biodegradable due to its polycyclic or heterocyclic aromatic structures (Provenzano et al. 2014). The binding of HS with hydrolytic enzymes may inhibit the hydrolysis efficiency of AD (Azman et al. 2017, Fernandes et al. 2014). From Figure 2, the concentration of HS in ALK12-ULS24 and ALK12 pretreated sludge was higher than that in ULS24 treated sludge. Hence, the large amount of refractory HS-like matters produced by ALK treatment may be another reason to slow down the initial biogas generation rate in ALK-related AD groups.

To investigate the correlation between DOMs and CMP, the Pearson's Correlation Coefficients were calculated and described (Table 3). CMP was strongly positive ($p < 0.01$) correlated to the concentrations of HMW PN, HMW PS, LMW neutrals and LMW acids. This reveals that the utilization efficiencies of these DOMs were higher in AD. Among them, LMW neutrals had the highest utilization efficiency ($R = 0.932$, $p < 0.01$) followed by HMW PS. In addition, the CMP had a stronger positive correlation with the concentration of HMW PS ($R = 0.859$, $p < 0.01$) than that with HMW PN ($R = 0.707$, $p < 0.01$). This further confirms that HMW PN had the relatively lower utilization efficiency in comparison with HMW PS. There were nearly no correlations between CMP and the concentrations of HS and building blocks, which is reasonable that HS-like substances are generally less biodegradable.

3.4 Identification of the residual DOMs after AD

The biochemical methane potential was in proportion to VS reduction (Table S2 and Table S3), implying the higher methane production consumed more organics, leaving less residual VS. But the residual soluble organics are determined by the composition and concentration of DOMs released by various pretreatment.

Sludge pretreatment releases both readily usable substance as well as some potential recalcitrant matters which may pass through AD process and be carried over to the subsequent wastewater treatment process. A significant amount of soluble residues were detected in the effluent from AD as shown in Figure 4, and the residues were much more in treated sludge AD effluent compared to those in control reactors’.

The distribution of residual DOMs from different digesters was similar (Figure 5a). The components of HS, HO DOC and LMW neutrals dominated the residual DOMs, which contributed 35.0%, 22.3%, 16.3% in control reactors and 32.9%, 26.6%, 13.6% in ALK12-ULS24 digesters, respectively. Moreover, the total amount of HS-like substances (HS and building blocks) occupied nearly half of the total residual DOMs. The majority of residual HO DOC, including hydrophobic HS, long chain alkanes and other HMW hydrocarbons, was refractory to hydrolysis or biodegradation. In addition, the amount of residual HMW PS in ALK12 pretreated sludge digesters was about 2 - 3 times higher than other reactors (Figure S4). Interestingly, the lower concentration of residual HMW PS in ALK12-ULS24 digesters indicates that the biodegradability of HMW PS produced by ALK pretreatment might be improved with ULS treatment.

It was found that LMW substances were the major compounds released by ALK12-ULS24 treatment and the components of LMW neutrals and biopolymers were more related to the biogas production in AD reactors. It would be interesting to investigate the detailed compounds profile and understand how these compounds were transformed in AD. GC-MS identified a total of 53 LMW (<580 Da) compounds with a similarity index above 80% in ALK12-ULS24 AD effluent, while 20 peaks were designated as “unknown”. The detailed profile is provided in Table S5 and Table S6. Similar types of compounds were found in digesters effluent with different pretreatment. The identified LMW DOMs were categorized as aromatics (mostly phenolic compounds),

alkanes, esters, acids, steroid-like matters, nitrogenated compounds (N-compounds, mostly amide compounds and amino acids) and others (alcohols and aldehydes) depending on the corresponding functional groups and properties.

In ALK12-ULS24 pretreated sludge digesters, there were a large amount of polycyclic steroid-like matters (45.4%) left over after AD, followed by alkanes (21.4%) and aromatics (10.1%) (Figure 5b). Among the above compounds, aromatics are generally more refractory to AD because of benzene rings. It seems ALK treatment may release a higher concentration of aromatics that led to higher aromatic residuals as well (Figure S5). Similarly, the higher concentration of alkanes produced in ALK12-ULS24 pretreatment may result in higher level of remaining alkanes. Further, N-compounds were also much more in ALK pretreated systems (Figure S5), indicating the serious fragmentation of cell membranes and other intracellular organic matters caused by alkali reaction. This may also increase the soluble N in the effluent if nitrogenated compounds were not easily degradable. In addition, the polycyclic steroid-like substances constituted nearly half of the residues in all the AD effluent. Nevertheless, the higher transformation of steroid-like substances in digesters with pretreated sludge implies that the pretreatment could promote the anaerobic utilization of these slowly biodegradable compounds.

Many compounds such as alkanes, acetophenone, phthalates, squalene, cholesterol and stigmasterol were identified at significant concentrations after pretreatment and then declined during AD process (Table S5 or Table S6). Phthalates were found to reduce from 1.28 mg/L to 0.10 mg/L in ALK12-ULS24 treated sludge during AD, indicating that phthalates may be intermediate products generated by pretreatment and could be gradually degraded anaerobically. Yuan et al. (2010) also observed the slow degradation of these compounds anaerobically. Cholesterol was

almost entirely reduced whereas cholestenol and coprostanol were still observed in the effluent. The latter two were likely the intermediates of cholesterol degradation (Jarde et al. 2005, Leeming et al. 1996). It is worthy to note that compounds like cis-11-Eicosenamide and glutarimide, N-(3-pentyl)- were observed in digested sludge while they were absent from both raw WAS and pretreated sludge samples. Further, their concentration was higher in the pretreated digested sludge compared to control systems. It is possible that these compounds were microbial by-products in AD.

Since GC-MS can only detect non-polar, relatively volatile and thermo-stable components, non-volatile compounds with higher molecular weight (< 2 kDa) were analyzed using UPLC-MS. The results validated that ALK12-ULS24 pretreatment promoted the release of compounds like amino acids, sugars, fatty acids, alkenes, steroids, sphingolipids, glycerophospholipids and their derivatives (Table S7). These compounds are mainly fragmentation of cytoplasm, extracellular substances and cell membrane. During AD process, most of them could be digested or transformed to other components as intermediate products. Thus, there were lots of soluble intermediate residues detected in the digested samples (Table S8), including polar metabolites (like dipeptide, phenylpropanoids, benzenoids and substituted derivatives), and lipids (like diacylglycerols, long chain fatty acids (LCFA), prenol lipids, alkenes, flavonoids, steroids, sphingolipids, glycerolipids, glycerophospholipids and their derivatives). Additionally, considerable amount of alkenes, LCFA, steroids and flavonoid were also found in pretreated samples (Table S7), suggesting they were generated during pretreatment and remained slow biodegradability or nonbiodegradability characteristics in AD.

One abundant group of residual compounds had simple chain structures, such as LCFA, alkenes, sphingolipids, diacylglycerols and glycerophospholipids (Table S8).

The number of identified lipids species in residues was more than that of polar metabolites, which may be due to their various homologues and the metabolism of anaerobic bacteria. The LCFA, produced during the hydrolysis or breakup of lipids, may become an inhibitor if the concentration is high. It is reported that LCFA was degradable but the degradation required longer retention time (Salminen and Rintala 2002).

The second abundant group of residues contained monocyclic or polycyclic structures (Table S8). For instance, the residual dipeptides were mainly the breakdown products of aromatic proteins, like tyrosine and tryptophan over other aliphatic proteins. Moreover, it has been well documented that organics like benzenoids and their derivatives were more recalcitrant and even inhibitory to cell growth and methanogenesis (Fernandez et al. 2017, Pham et al. 2013). Besides, phylloflavanine, classified as flavonoid, was proposed to be refractory with multiple complex benzene rings and double bonds. Citreoviridin C (classified as Pyranones and derivatives) was recognized as mycotoxin, which may inhibit the activity of bacterium. These compounds once accumulated in the biological systems can cause serious inhibition. To date, it is still not clear how the recalcitrant compounds would be transformed in the downstream wastewater treatment facilities.

This study found out that the pretreatment caused a significant solubilization of sludge, leading to the great increase of soluble HMW PN, HS-like matters, LMW neutrals and HMW PS. During AD, LMW neutrals had the highest digestion efficiency, followed by HMW PS and HMW PN. It implies LMW neutrals, HMW PS and HMW PN were more correlated with the biogas generation. HS-like matters, on the other hand, were nearly nonbiodegradable, and remained in the residues. It should be noted that the solids that were not solubilized in the pre-treatment step may still contribute to the

biogas production after long-term digestion, while it is challenging to discuss the correlation between biogas production and solubilization of this particular part of solids.

This study also found out that identification and quantification of produced DOMs from pre-treatment can be a cost-effective and time-saving method to predict the digestion efficiency of sludge. This study can help to better understand the mechanisms of various pretreatment methods, and assist in the design of more effective sludge treatment and post-treatment processes.

4. Conclusions

Combined ultrasonication and alkaline pretreated sludge displayed the best performance in terms of solids solubilization and methane production. However, more refractory substances, such as HS-like matters and HO DOC also remained after AD process. The pretreatment promoted the release of biodegradable organic matter as well as recalcitrant HS and complex HMW PN, which were proved to be hardly biodegradable. LMW residual DOMs mainly composed of polycyclic steroid-like matters, alkanes and aromatics which accounted up to 45.4%, 21.4% and 10.1% of total residual DOM, respectively. HMW residues included polar metabolites and non-polar lipids, among which phylloflavanine and mycotoxin of Citreoviridin C were identified as recalcitrant and/or inhibitory compounds. It is recommended that further treatment step for anaerobic digester liquor should be considered to reduce these refractory residuals.

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634
635

List of Tables

- Table 1.** Characteristics of WAS and seed sludge.
- Table 2.** Pearson's Correlation Coefficients (*R*) between pretreatment parameters and DOMs compositions.
- Table 3.** Pearson's Correlation Coefficients (*R*) between cumulative methane production (CMP) and the available DOMs.

Table 1. Characteristics of WAS and seed sludge.

Parameters	WAS (g/L)	Seed sludge (g/L)
Total solids (TS)	13.62 ± 0.15	15.44 ± 0.23
Volatile solids (VS)	10.81 ± 0.20	11.23 ± 0.11
Total suspended solids (TSS)	12.45 ± 0.14	14.27 ± 0.22
Volatile suspended solids (VSS)	10.36 ± 0.18	10.62 ± 0.28
tCOD	15.01 ± 0.23	15.52 ± 0.31
sCOD	0.25 ± 0.10	0.26 ± 0.13

Table 2. Pearson's Correlation Coefficients (*R*) between pretreatment parameters and DOMs compositions.

	DD	HI	HMW	HMW	Bio	HS	Building	LMW	LMW
		DOC	PN	PS	polymers		Blocks	Neutrals	Acids
pH	0.466 [*]	-	-	-	-	-	-	-	0.616 ^{**}
SEI	0.792 ^{**}	0.764 ^{**}	0.917 ^{**}	0.602 ^{**}	0.904 ^{**}	-	-	0.751 ^{**}	-

^{**}Correlation is significant at the 0.01 level (2-tailed).

^{*} Correlation is significant at the 0.05 level.

“-” denotes correlation is insignificant ($p > 0.05$).

Table 3. Pearson's Correlation Coefficients (*R*) between cumulative methane production (CMP) and the available DOMs.

	HI DOC	HMW PN	HMW PS	Bio polymers	HS	Building Blocks	LMW Neutrals	LMW Acids
CMP	0.868**	0.707**	0.859**	0.824**	-	-	0.932**	0.785**

**Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level.

“-” denotes correlation is insignificant ($p > 0.05$).

List of Figures

- Figure 1.** Effects of pH and specific energy input (SEI) on EPS fractions.
- Figure 2.** Quantification of sub-fractions (mg/L -C) of DOMs after pretreatment.
- Figure 3.** Cumulative methane production (CMP) curves of control, ALK12, ULS24 and combined ALK12-ULS24 pretreated sludge. A: Enlargement of the first 10 days. B: Methane production profile in the 30 days operation.
- Figure 4.** Transformation of DOMs during AD for control and ALK12-ULS24 sludge digesters. Comb. denotes ALK12-ULS24 sludge digesters, Cont. denotes control digesters.
- Figure 5.** (a) The distribution of residual DOMs. (b) The distribution of specific residual LMW DOMs (< 580 Da) after AD.

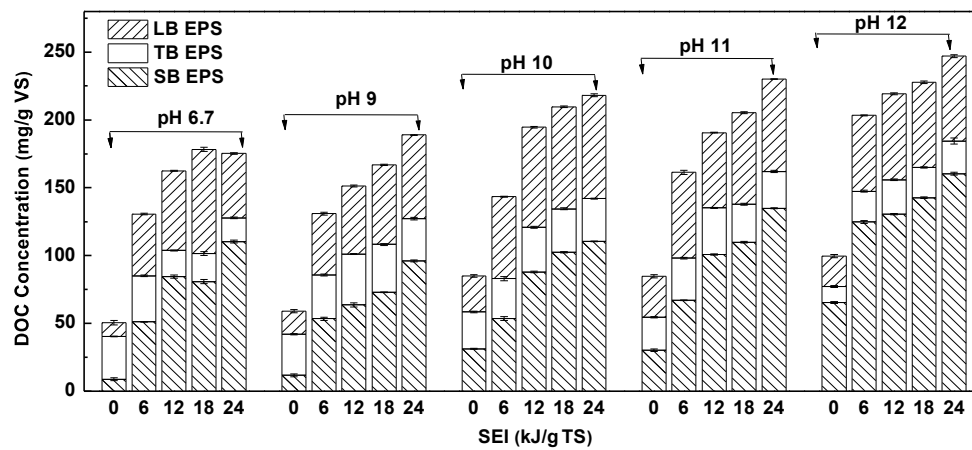


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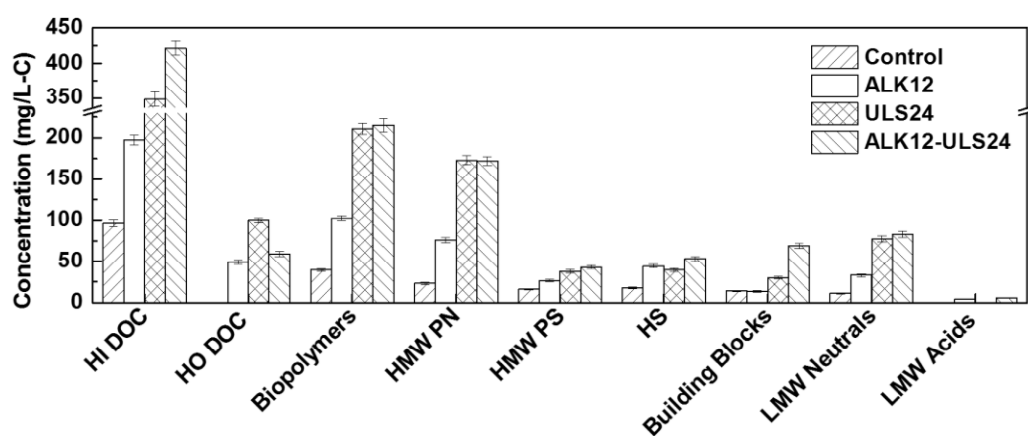


Figure 2. Quantification of sub-fractions (mg/L -C) of DOMs after pretreatment.

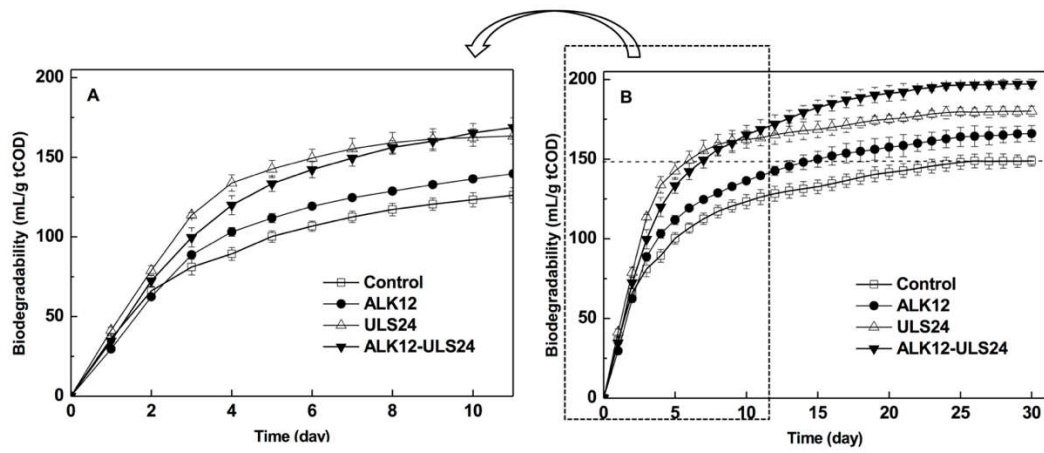


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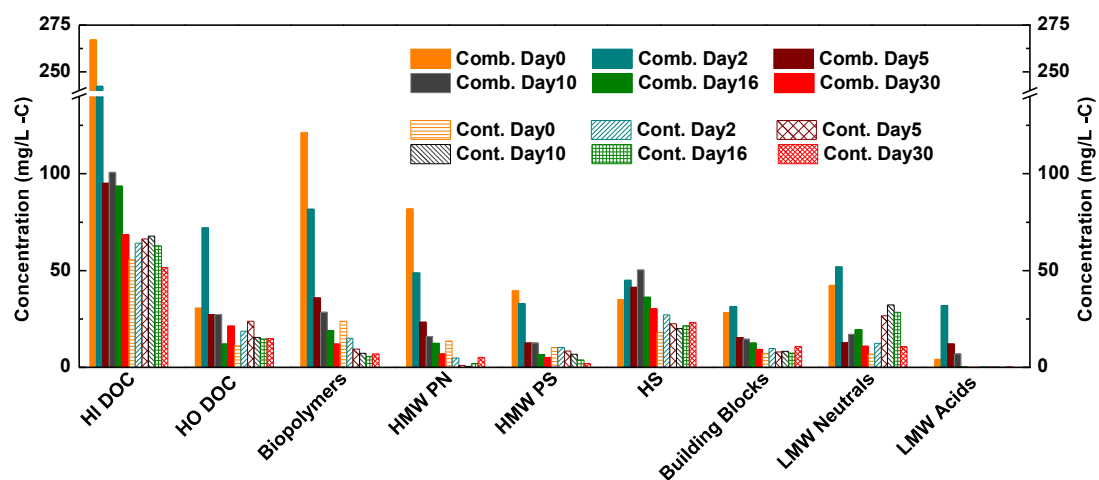


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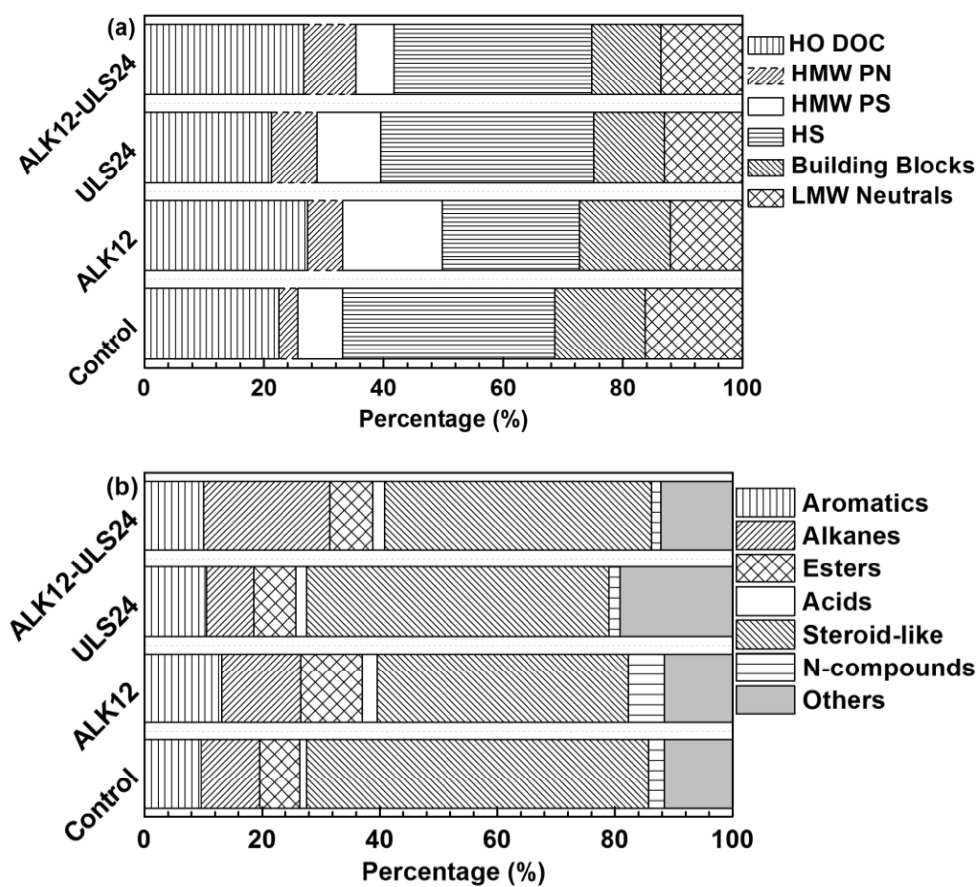


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