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Molecular and microbial insights towards understanding the anaerobic digestion of the wastewater from hydrothermal liquefaction of sewage sludge facilitated by granular activated carbon (GAC)

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ABSTRACT

Hydrothermal liquefaction of sewage sludge to produce bio-oil and hydro-char unavoidably results in the production of high-strength organic wastewater (HTLWW). However, anaerobic digestion (AD) of HTLWW generally has low conversion efficiency due to the presence of complex and refractory organics. The present study showed that granular activated carbon (GAC) promoted the AD of HTLWW in continuous experiments, resulting in the higher methane yield (259 mL/g COD) compared to control experiment (202 mL/g COD). It was found that GAC increased the activities of both aceticlastic and hydrogenotrophic methanogens. The molecular transformation of organics in HTLWW was further analyzed. It was shown GAC promoted the degradation of soluble microbial by-products, fulvic- and humic-like substances as revealed by 3-dimensional fluorescence excitation-emission matrix (3D-EEM) analysis. Gas chromatography mass spectrometry (GC–MS) analysis showed that GAC resulted in the higher degradation of N-heterocyclic compounds, acids and aromatic compounds and less production of new organic species. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis also showed that GAC promoted the degradation of nitrogenous organics. In addition, it was shown that GAC improved the removal of less oxidized, higher nitrogen content, and higher double bond equivalent (DBE) organic compounds. Microbial analysis showed that GAC not only increased the microbial concentration, but also enriched more syntrophic bacteria (e.g., *Syntrophorhabdus* and *Synergistes*), which were capable of degrading a wide range of different organics including nitrogenous and aromatic organics. Furthermore, profound effects on the methanogens and the enrichment of *Methanothrix* instead of *Methanosarcina* were observed. Overall, the present study revealed the molecular transformation and microbial mechanism in the AD of HTLWW with the presence of GAC.

1. Introduction

The proper management of dewatered sewage sludge (DSS) is crucial in municipal wastewater treatment plant (WWTP). Currently, more than 8 million tons/year DSS are produced from 4000 domestic WWTPs in China (Yin et al., 2018). DSS is affluent in organics and nutrients, which could contaminate the environment if not properly treated. Alternatively, it can be used as an energy source (Liu et al., 2018), and therefore different technologies (e.g., chemical, biological, and thermal technologies) have been developed to produce bioenergy from DSS.

Hydrothermal liquefaction (HTL) is a thermochemical technology, which can convert wet biomass into liquid (bio-oil) and solid (hydro-char) biofuels (Cheng et al., 2018; Si et al., 2019; Usman et al., 2019). It is a promising technology and has been used for the conversion of DSS

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(Chen et al., 2019). However, a large quantity of aqueous phase, namely hydrothermal liquefaction wastewater (HTLWW), is generated as a by-product during HTL process. This high-strength wastewater generally contained 20–40% organics and 60–80% nutrients of the feedstocks (Li et al., 2017; Nie and Bi 2018; Usman et al., 2019; Zhang et al., 2018b). Therefore, the valorisation of HTLWW is a censorious step for maximizing the nutrient and energy recovery from the HTL process to make the whole process economical and environment-friendly (Li et al., 2017; Usman et al., 2019).

Anaerobic digestion (AD) has been widely used for the conversion of organic wastes into biogas. The components of HTLWW are very complex, including biodegradable organics (organic fatty acids, sugars) and resistant and toxic organics (N-heterocyclic compounds, phenol, furan and their derivatives) (Maddi et al., 2016; Sudasinghe et al., 2014; Zhu et al., 2016). The transformation of HTLWW organics during AD process was reported previously (Si et al., 2018; Tommaso et al., 2015; Zheng et al., 2017), and generally 33–64% of the organics in HTLWW could not be converted (Usman et al., 2019).

The performance of AD of HTLWW needs to be upgraded to make the process more economically feasible. Previous studies have explored different methods to increase the methane yields of HTLWW (Chen et al., 2017; Si et al., 2019; Tommaso et al., 2015; Zheng et al., 2017; Zhou et al., 2015). Ozone was used to degrade the resistant and toxic organics, which increased the methane yield of HTLWW of swine manure by 109% (Si et al., 2019). The removal of resistant and toxic organics from HTLWW could also be achieved by using organic solvent extraction (Chen et al., 2016) and adsorbents (zeolite, polyurethane matrices, granular activated carbon (GAC) (Zheng et al., 2017) and powder activated carbon (Zhou et al., 2015)), which could also increase the anaerobic degradability of HTLWW by AD. For instance, several studies used GAC to adsorb the organics in HTLWW before AD, and the COD can be removed as high as 93% (Si et al., 2019; Yang et al., 2018; Zheng et al., 2017; Zhou et al., 2015). Instead of using GAC as adsorbent, recent studies also showed that GAC could promote the biogas production efficiency from sewage sludge, glucose and short chain fatty acids due to the possibility to promote direct interspecies electron transfer (DIET) (Barua and Dhar, 2017). However, it has not been tested for HTLWW, which contains complex soluble organics. Although most previous studies used batch experiments to eveluate the effects of GAC on methane production from sludge or glucose, it is not suitable for HTLWW because it is difficult to exclude the effects of adsorption of inhibitory organics by GAC on methane production. Continuous experiments would be more appropriate because GAC would be saturated after some time and microbes could be well established on GAC after long-term operation.

The components of HTLWW were fairly complex with a wide range of molecular weights. Previous studies mainly focused on qualitative analysis with gas chromatography-mass spectrometry (GC–MS) but most of the high molecular weight (HMW) compounds cannot be vaporized into the column and still remain uncharacterized (Cao et al., 2017; Wirth et al., 2015). Very few publications have provided insight into the molecular structures of HMW compounds in HTLWW as well as the relationships between the molecules and GAC during the continuous experiments. The combination of different analytical methods is necessary to give better understanding of the organic compositions in HTLWW. For instance, Fourier transform ion cyclone resonance mass spectrometry (FT-ICR-MS) can help us unveil the detailed molecular properties in biogas reactors considering its ultrahigh resolution and mass accuracy (Kamjunke et al., 2017; Lu et al., 2018).

Based on the above considerations, the current study aimed to investigate the biogas production from HTLWW in the presence of GAC in the continuous experiments. The degradation of organics was characterzied by a suit of different technologies including FT-ICR-MS, GC–MS, and 3-dimensional fluorescence excitation-emission matrix (3D-EEM) spectroscopy. Moreover, the microbial communities in the biofilm on the surface of GAC as well as the suspened sludge in the liquid were investigated by high-throughput sequencing of 16S rRNA genes. These results would provide an in-depth understanding on the effects of GAC on the AD of HTLWW from molecular and microbial aspects.

2. Material and methods

2.1. HTL of DSS

HTL was conducted by using a 3 L stainless steel autoclave reactor. DSS was collected from a wastewater treatment plant in Shanghai, China, and the moisture content of DSS was 84.5%. The reaction temperature was set at 300 °C and kept for 1 h in the autoclave, and the HTL condition was commonly used for bio-oil production through HTL of biomass (Li et al., 2017; Sudasinghe et al., 2014). 2 L sludge was loaded in the reactor, closed and heated up from the room temperature to the desired temperature with the heating rate of 5 °C/min. During the whole HTL, the agitation was kept at 500 rpm. The vessel was quenched rapidly to room temperature via internal cooling water circulation upon completion of reaction.

The solid and liquid products were centrifuged at 5000 rpm for 2 min. Liquid was stored in a glass bottle at −20 °C for further analysis. The characteristics of HTLWW are shown in Table S1.

2.2. AD of HTLWW in continuous experiments

The continuous experiments were conducted by using two 1 L reactors with working volume 600 mL. The reactors were operated as anaerobic sequencing batch reactor (ASBR). The COD of HTLWW was kept at 10 g/L by six times dilution of the raw HTLWW with tap water and sustained throughout the whole process. The pH of HTLWW was adjusted to 7.5 by using 2 M NaOH and HCl solution, considering the high pH (8.7) of HTLWW that was not optimal for anaerobic digetison. The experiments were performed at mesophilic condition (37 °C) in water bath system. Initially, both reactors were inoculated with 200 mL inoculum with 400 mL HTLWW at stirring speed 200 rpm. The inoculum was collected from the biogas reactor treating sewage sludge in a wastewater treatment plant (Shanghai, China). GAC (10 g/L) was added to one of the reactor, denoted with “ASBRG” and the other reactor was used as control and denoted with “ASBRC”. Both reactors were flushed with nitrogen (N_2) for 5 min to eliminate air and sealed with lids. Initially, the reactors were run in batch modes until there was no obvious biogas production. Then, both reactors were run in continuous modes. Hydraulic retention time (HRT) of both reactors was maintained at 4 days. The ASBR cycle was 1 day, and it was settled for 1 h before drainage and then fed with HTLWW. The produced biogas was collected in gas bags.

2.3. Specific methanogenic activities of acetate and H_2/CO_2

Sludge samples were obtained from the two ASBRs during the steady states, and then they were used to evaluate the specific methanogenic activities by using acetate (30 mM) and H_2/CO_2 (80/20, 1 atm) as substrates by batch experiments, respectively. 5 mL samples were transferred to 20 mL serum bottles, and then acetate and H_2/CO_2 were added to the bottles. The bottles containing reactor samples (Without substrates) were used as controls. All the bottles were flushed with nitrogen for 5 min and kept in an incubator at 37 °C with shaking speed 200 rpm. The experiments were conducted in triplicates.

2.4. Microbial analysis

Microbial samples were collected during steady states of the two ASBR reactors. The reactors were well mixed to get samples for quantification of microbial concentrations in both reactors by qPCR. The suspended sludge from the two reactors, and also the attached biofilm

on GAC were collected for the analysis of microbial community compositions. Duplicate samples were obtained during the steady-states. Total genomic DNA was extracted from each sample using QIAamp DNA Stool Mini Kit (QIAGEN, 51504). The amount and quality of the extracted DNA were detected by Nanodrop (2000). qPCR was then conducted with the universal primers 515 F (5′-GTGCCAGCMGCCGC GGTAA-3′) and 806 R (5-GGACTACHVGGGTWTCTAAT-3′) targeting both bacteria and archaea. The qPCR conditions and calculation of the 16S rRNA genes were based on a previous study (Ding et al., 2016). qPCR were run in triplicate with the DNA extracted from each sample. For the analysis of microbial community compositions, PCR was also conducted with the universal primers 515 F and 806 R. The PCR products were purified, quantified and then used for barcoded libraries preparation and sequenced on an Illumina Miseq platform according to the standard protocols. The raw sequences were submitted to NCBI with accession number SUB5714188. The bioinformatics analysis of high-quality sequences were based on our previous study (Chen et al., 2019).

2.5. 3D-EEM spectroscopy analysis

During the steady-states of both reactors, liquid samples of the influent and effluents were also collected for the characterization of the organics. To analyse and differentiate the different types and sources of dissolved organic matters (DOM), 3D-EEM analysis was performed at emission wavelength 280–600 nm and excitation wavelength 250–450 nm. The fluorescence intensity peaks was evaluated by applying parallel factor (PARAFAC) analysis model technique based on previous studies (Chen et al., 2017; Maie et al., 2014).

2.6. GC–MS analysis

The organic compositions of the liquid samples were identified by using GC–MS (Focus DSQ, Termoelectron, America). Gas chromatography was executed on a 30-m HP-INNOWax quartz capillary column with inner diameter (0.25 mm) and film thickness (0.25 μm) with injection temperature of 250 °C. Initially, the column was hold at 60 °C for 2 min and then heated up to 250 °C and maintained for 10 min. Helium gas was used as the carrier gas with flow rate 1.0 mL/min. The chemical identification was performed by NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) Mass Spectral Database.

2.7. FT-ICR-MS analysis

The liquid samples were also examined by a Bruker Apex ultra FT-ICR MS equipped with a 9.4 T superconducting magnet. The samples were diluted with methanol solution and injected into the electrospray source at 180 μL/h using a syringe pump. The examination involved a 3.5 kV spray shield voltage, 4.0 kV capillary voltage, and −320 V capillary column end voltage. Ions accumulated in the ion source for 0.001 s in a hexapole. Ions passed through a quadrupole, accumulated in an argon filled hexapole collision pool, in which ions accumulated for 0.2 s. The delay was set to 1.0 ms to transfer the ions from the collision poll to an ICR cell by electrostatic focusing of transfer optics. The mass range was set at m/z 150–600. The methodologies used for FT-ICR MS mass calibration, data acquisition, and processing were described elsewhere (Liu et al., 2010; Yuan et al., 2017).

2.8. Other analysis

pH of HTLWW samples were measured by using pH meter (FE20, Mettler Toledo, Switzerland). Chemical oxygen demand (COD), Total Nitrogen (TN) and ammonia nitrogen were measured according to APHA (American Public Health Association, 1998). Total organic carbon (TOC) was analysed by a TOC analyser (TOC-L CPH, Shimadzu, Japan). The gas composition was detected by gas chromatography (GC 960) coupled with thermal conductivity detector. The helium was used as the carrier gas and the temperatures of the injector, detector and oven were 120 °C, 110 °C and 120 °C respectively (Liu et al., 2016). Volatile fatty acids (VFA) were analysed by using high-performance liquid chromatograph GC (Shimadzu G2010) with a fame ionization detector. The detailed information about VFA analysis was mentioned in our previous studies (Chen et al., 2016; Tommaso et al., 2015).

Table 1
Characteristics of HTLWW and summary of the reactors performances during steady states of both reactors.

Parameters	Influent	ASBRC	ASBRG
pH	7.5 ± 0.1	7.85 ± 0.2	7.9 ± 0.1
COD (mg/L)	10100 ± 120	4200 ± 150	2600 ± 160
NH_4^+-N (mg/L)	1360 ± 115	1310 ± 108	1282 ± 94
TOC (mg/L)	2970 ± 122	1450 ± 107.8	980 ± 4.2
CH_4 yield (mL/g COD)	–	202 ± 11	259 ± 11
Acetic acid (mg/L)	822 ± 12	8.5 ± 1.8	4.6 ± 0.6
Propionic acid (mg/L)	269.7 ± 4.1	1.9 ± 0.1	1.6 ± 0.2
i-butyric acid (mg/L)	150.2 ± 2.9	1.4 ± 0.1	1.5 ± 0.1
n-butyric acid (mg/L)	265.7 ± 5.2	1.5 ± 0.4	0.4 ± 0.1
i-valeric acid (mg/L)	267.1 ± 5.4	2.8 ± 0.3	3.60 ± 0.2
n-valeric acid (mg/L)	117 ± 2.1	2 ± 0.2	1.20 ± 0.2

3. Results and discussion

3.1. Effect of GAC on CH_4 production and organics conversion

Both ASBR reactors were operated for more than 80 days until steady-state was achieved (Fig. S1), and the performances of the reactors are summarized in Table 1. The COD removal efficiency of ASBRC was 58%, while it was increased to 74% in ASBRG. Correspondingly, the methane yield was increased from 202 mL/g COD in ASBRC to 259 mL/g COD in ASBRG. The above results clearly showed that GAC promoted the methane production from HTLWW. The pH in both reactors were 7.9 and higher than that of the influent (7.5), which could be attributed to the consumption of VFA in the raw HTLWW. The concentrations of ammonia in both reactors were not obviously affected by GAC. The VFA in the effluent of both reactors were very low (< 10 mg/L), indicating that they could be well converted to methane in the reactors and methenogenesis was not a limiting step in the current study. Previous studies showed that GAC promoted VFA conversion rates during the sludge digestion, and thereby increased the overall conversion efficiency of sludge (Yang et al., 2017; Zhao et al., 2017). Considering the low VFA concentrations in both reactors, GAC might promote the degradation of other hard-biodegradable organics, and thereby increased the overall COD removal efficiency.

The specific methanogenic activities of the enriched mixed cultures in ASBR reactors on acetate and H_2/CO_2 were further investigated. A significant difference was observed in Fig. S2, and the CH_4 production rates from both acetate (28 mL/gVS/d) and H_2/CO_2 (22 mL/gVS/d) by the sludge from ASBRG were much higher than those (18 mL/gVS/d for acetate and 15 mL/gVS/d for H_2/CO_2) from ASBRC. It was known that acetate and H_2/CO_2 were the two main intermediates for methanogenesis (Zhang et al., 2018a). Therefore, GAC promoted the rates of both hydrogenotrophic and acetoclastic methanogenesis. Previous studies suggested that GAC could enhance methane production rate through DIET, and the specific H_2 consumption rate could not be increased because the enriched cultures preferred to use H^+ instead of H_2 (Barua and Dhar, 2017; Jing et al., 2017). In the present study, it seems GAC promoted interspecies H_2 transfer considering the increased H_2 consumption rate. The increased H_2 consumption rate was related to the increased COD removal by the enriched microbes, which provided more H_2 as substrates through IHT. Therefore, subsequent investigation is necessary to elucidate the role of GAC in AD of HTLWW from molecular and microbial aspects.

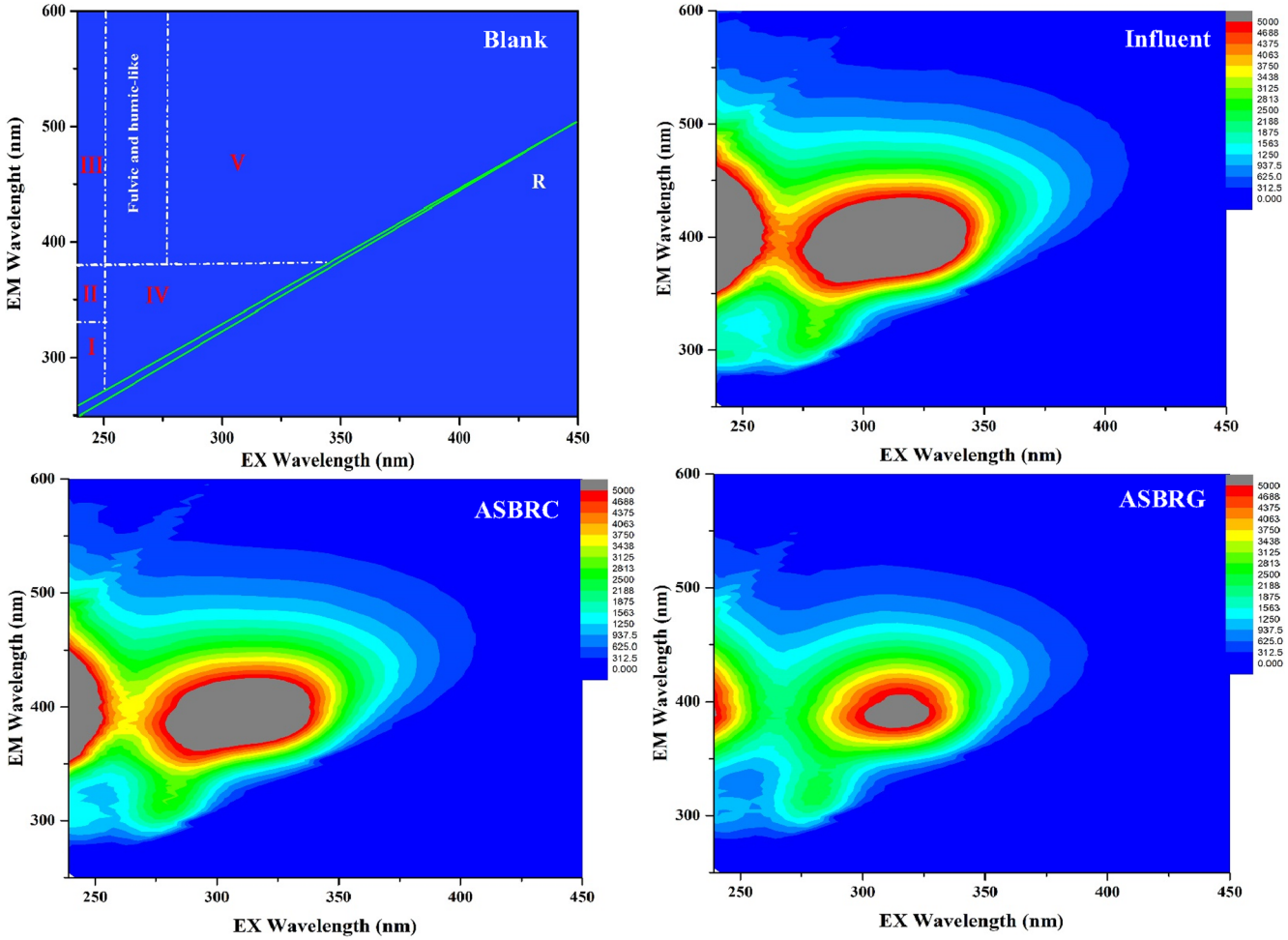


Fig. 1. The changes of fluorescent components in HTLWW by AD as revealed by 3-EEM spectrum.

3.2. Effect of GAC on the degradation of fluorescent components in HTLWW

The fluorescent compounds in the influent and effluent samples of both reactors were characterized by 3D-EEM (Fig. 1). The PARAFAC analysis identified three components (Figs. S3a and S3b). Component 1 (C_1) had maximum Ex/Em peak at 278/380 and was associated with soluble microbial by-product substances. Component 2 (C_2) had two peaks with the first peak at 275/295 (Ex/Em) and second peak at 373/415 (Ex/Em), which were related with fulvic- and humic-like substances. The third component (C_3) had only one peak at 320/386 Ex/Em wavelength which was linked with humic-like substances (Carstea et al., 2016; Chen et al., 2019; Oloibiri et al., 2017). The soluble microbial by product (C_1) could result from microbes during substrate metabolism (Azami et al., 2012; Li et al., 2013). The presence of fulvic- and humic-like substances could be due to polymerization and condensation reactions during HTL of DSS (Guo et al., 2019; Qi et al., 2019). C_1 was dominant in the influent, and it was reported to be biodegradable compounds. The decrease of C_1 was observed in both reactors, while ASBRG achieved higher removal efficiency of such component (13.9% in ASBRC and 50.4% in ASBRG) (Fig. 2). Although C_2 and C_3 were related with fulvic and humic-like substances, which were reported to be non-biodegradable compounds, they were found to be degraded to a certain extent in both reactors due to some specific types of microbial enrichment (Lee and Hur, 2016; Qi et al., 2019; Xue et al., 2012). In fact, a previous study also showed certain compounds present in the humic-like region were biodegradable and not refractory in nature (Oloibiri et al., 2017). ASBRG also showed higher removal efficiency of such components. The above results clearly showed that GAC promoted the degradation of fluorescent compounds (e.g., soluble microbial by-products, fulvic- and humic-like substances), which resulted in the higher methane production.

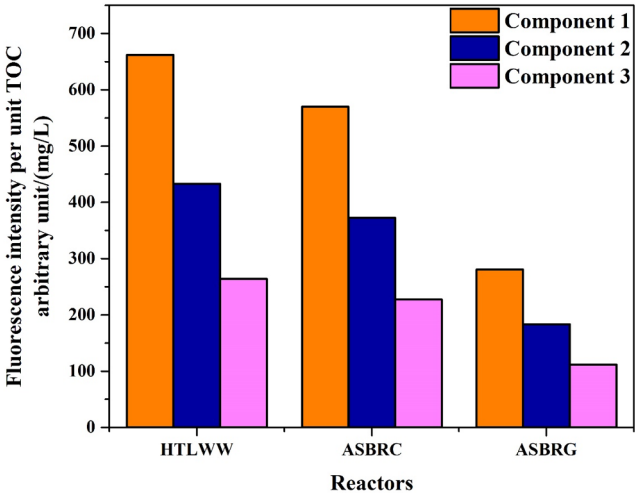


Fig. 2. Fluorescence intensity of the EEM- PARAFAC C1-C3 components in HTLWW and AD effluents.

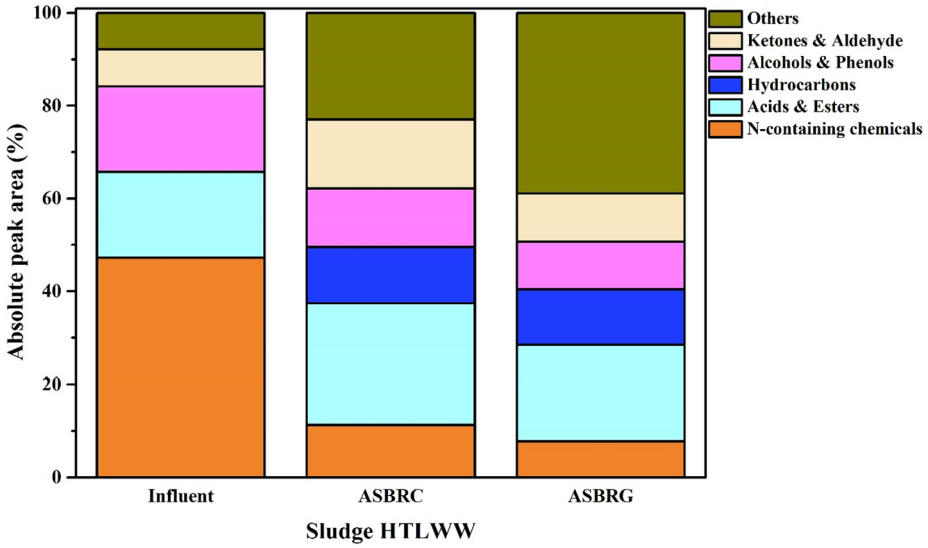


Fig. 3. Organic compositions in HTLWW and AD effluents by GC–MS analysis.

3.3. Effect of GAC on organic compositions in HTLWW as revealed by GC–MS analysis

GC–MS analysis was further performed to qualitatively detect the organic compounds in the liquid samples. As illustrated in Fig. S4, the peak numbers and intensities declined remarkably after the AD process. There were 29 organics detected in the HTLWW (Influent) as summarized in Table S2. After AD, 23 organics disappeared and were supposed to be biodegradable compounds and could not be detected in both ASBRC and ASBRG. The organics were classified into five major subcategories such as nitrogenous compounds, acids & esters, hydrocarbons, alcohol & phenols, ketones & aldehydes, and others compounds (chemicals had less than 1% area), and illustrated by absolute peak area variation based on GC–MS analysis in Fig. 3. Nitrogenous compounds were dominant in the influent, which could be due to the degradation of proteins during HTL of DSS (Chen et al., 2019; Usman et al., 2019). However, it was no longer the dominant compounds after AD, indicating higher removal efficiency of nitrogenous compounds during AD. The results of semi-quantitative analysis based on peak intensity are shown in Tables 2 and S2. Pyrazine, 2-ethyl-3-methyl-; piperidine, 3,5-dimethyl-; N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid and oxacyclododecan-2-one were removed with high efficiency (> 95%), while digitoxin and retinal, 9-cis- were not well removed. However, GAC promoted the degradation of organics by 12.3% (based on absolute total peak area) in ASBRG comparing with ASBRC, which could be due to the enrichment of certain microbes.

Some organic compounds not detected in the influent were found in the effluents, and the newly generated organics are shown in Table 2. There were 14 and 10 types of new organic compounds appeared in ASBRC and ASBRG, respectively, including 6 kinds of shared compounds. These new pollutants may be produced as intermediates from the degradation of aromatics and branched chain structure chemicals, which were present in the influent, into the straight and short chain hydrocarbons through deoxygenation during AD process (Abdel-Shafy and Mansour, 2018; Thi Nhi Cong, 2008). For example, octadecane, 1-isocyanato-, undecanoic acid, γ-dodecalactone and 2-dodecanone were present in the influent with high intensity, while these compounds were fully removed and converted into penta, hexa, hepta and octadecane hydrocarbons with larger peak spectrum after AD (Table 2). The lower residual aromatic compounds intensity in ASBRG might result in high CH_4 yield because aromatic compounds were refractory chemicals for AD process (Leng and Zhou, 2018; Tommaso et al., 2015; Usman et al., 2019). The above results showed that GAC promoted the degradation of N-heterocyclic, acids and aromatic compounds by complete or partial conversion. The addition of GAC also converted some specific types of aromatic compounds into straight chain hydrocarbons and produced less new organic species.

3.4. Effect of GAC on organic compositions in HTLWW as revealed by FT-ICR-MS analysis

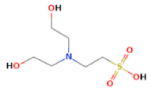
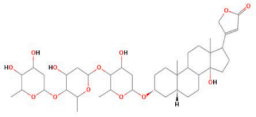
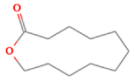
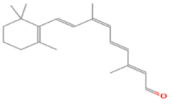
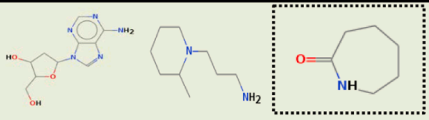
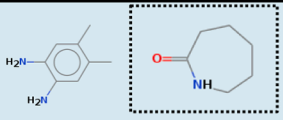
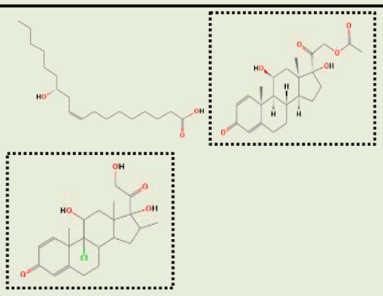
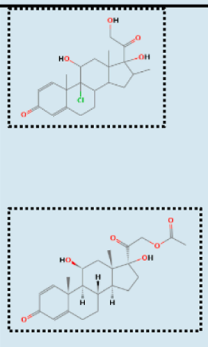
As GC–MS could not detect non-volatile compounds with high boiling points (a large portion of organics in HTLWW), ESI FT-ICR-MS technique was used to gain a broader insight of the organic transformation of HTLWW during the AD process. ESI FT-ICR-MS spectrums show that the MW distribution of the influent and two effluents ranged from 200 to 600 Da (Fig. S5) (Sudasinghe et al., 2014). Thousands of molecular formulas were assigned to the spectrums and the molecular characteristics were evaluated based on these formulas (Altieri et al., 2009; Headley et al., 2014). According to Table S3, the effluents exhibited more diversity of components than the influent as shown by the total molecule numbers, which increased from 1166 in HTLWW to 2323 and 1822 in the effluents of ASBRC and ASBRG, respectively. It was consistent with GC–MS results that fewer new organic species were detected in ASBRG compared to ASBRC (Table 2). This may be attributed to the degradation of large molecules into more small components during AD. A recent study also described the presence of higher MW chemicals in HTLWW through HTL of swine manure (Si et al., 2019). As the influent derived from sludge contains abundant nitrogenous organics, it is reasonable that the CHON compounds were the predominant component (> 50%) in the influent and effluents (Table S3).

Fig. 4 shows the MW distribution of both CHO and CHON compounds in the influent and effluents. After AD, the relative abundance of molecules with low MW (250–400 Da) reduced while molecules with high MW (400–600 Da) showed the opposite trend. It might be due to the high bioavailability of small molecules during AD. The molecules with low MW were degraded faster than high MW, which led to the accumulation of high MW molecules and their higher relative abundance in the effluent. The low mass range indicated the presence of hydrolysis products from macromolecules (> 1000 Da) and these organics could be oligomers, which were also observed in HTLWW from algae (Maddi et al., 2016). The addition of GAC tended to affect CHON compounds more than CHO compounds in terms of MW distribution by comparing the ASBRC and ASBRG effluents. The addition of GAC in the bioreactor probably promoted the degradation of more CHON compounds with high MW, which was supported by the much lower relative

abundance of high MW region in the ASBRG effluent compared with ASBRC effluent. Similar trend was also noted in GC–MS analysis for degradation of nitrogenous compounds in both reactors (Fig. 3).

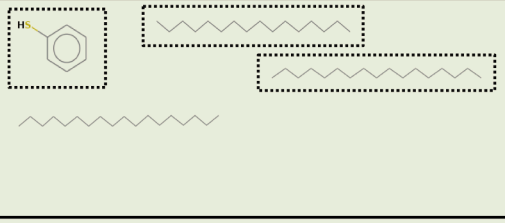
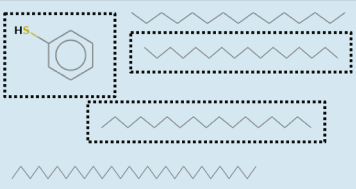
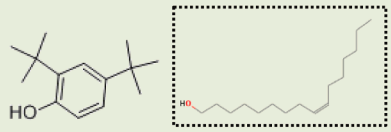
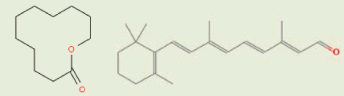
Previous studies classified the molecules into several categories (e.g., lipids and aliphatic/proteins) with different H/C and O/C ranges (Bianco et al., 2018; Geng et al., 2018; Shakeri Yekta et al., 2012). The van Krevelen (VK) diagram (Fig. 5) revealed the changes of CHON and CHO compounds after AD with and without GAC. As the influent was obtained from HTL of sludge, which mostly consisted of protein, the enriched CHO or CHON components (i.e., big bubble in the influent plot) were mostly likely CRAM-like compounds. The top three plots for CHO compounds (Fig. 5) showed more diverse components in both ASBRG effluent and ASBRC effluent than in the influent. This indicated the enriched components in the influent were degraded after AD and more small components were produced with more diverse distribution of O/C and H/C. Similar trend was observed for CHON compounds, showing the capacity of microbes to degrade CHON components (Shakeri Yekta et al., 2012). The difference of CHO compounds between the ASBRG effluent and ASBRC effluent from the prospective of VK diagram were subtle, which might suggest that GAC addition had a minor effect on the degradation of CHO compounds. The aforementioned MW change of CHO and CHON compounds also confirmed this point. However, for CHON compounds, a small portion of unsaturated hydrocarbons was found in the GAC effluent. GC–MS results also

Table 2
GC–MS analysis of the removed and newly generated organics.

Influent		**ASBRC**	**ASBRG**
Removal efficiency of certain organics (%)		98.24	98.61
		98.27	98.60
		96.52	97.32
		13.61	17.72
		95.60	95.99
		49.56	55.54
Newly generated pollutants at different subcategories [a]	***Nitrogen containing compounds***		
	Acids & Esters		

(*continued on next page*)

Table 2 (*continued*)

	Hydrocarbons		
	Alcohols & phenols		
	Aldehyde & ketone		
	Total new pollutant types	14	10

[a]: The appearing of similar organics in ASBRC and ASBRG denoted with dotted box

revealed the high amount of hydrocarbons occurrence in ASBRG compared with ASBRC. It might be possibly due to some specific type of microbial enrichment during the AD.

The removed, resistant, and produced compounds in both reactors are shown in Fig. 6. The removed compounds referred to the molecules that only presented in the influent and disappeared in the effluent. Fig. 6A and B showed that the addition of GAC slightly affected the removal of CHO compounds while significantly affected CHON compounds. The addition of GAC tended to remove more CRAM-like compounds. The resistant compounds were the molecules in both influent and effluents while the intensity might change during the process. Therefore, these molecules cannot be taken as the completely resistant compounds. Their presence in the effluent might be due to the residue of parent compounds after partial degradation or transformation of other compounds. The produced compounds were the new molecules only present in the effluent. The right plot of Fig. 6 exhibited that the addition of GAC facilitated to produce lower O/C compounds at unsaturated hydrocarbon area. This might indicate that the microbes enriched by GAC produced less oxidized substances. As shown in Fig. S6, semi-quantitative analysis of different types of compounds showed that the degradation of aliphatic/protein compounds decreased by 4–6% on the difference of relative intensity after AD. The lower percentage of aliphatic/protein area for the ASBRG effluent compared with the ASBRC effluent indicated that the addition of GAC promoted more degradation of aliphatic/protein compounds (Fig. 6). The higher percentage of CRAM-like area might be due to the slower degradation compared with aliphatic/protein compounds or metabolite of microbes. It was reported that the molecular weight of carboxylic-rich alicyclic

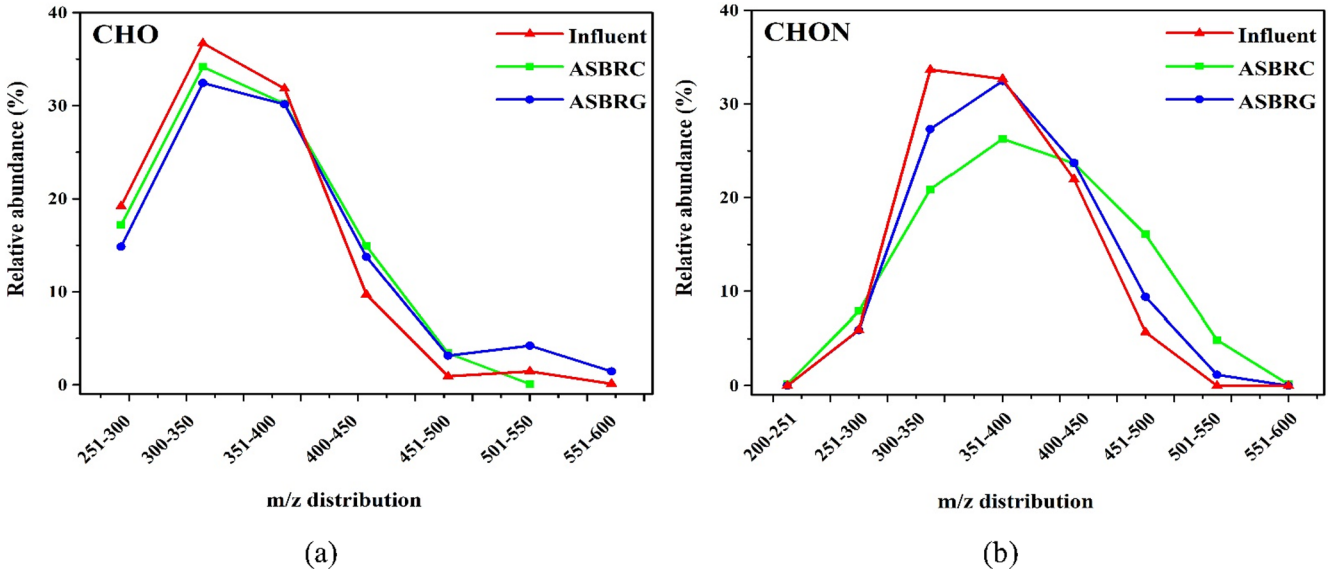


Fig. 4. Molecular size distribution of (a) CHO compounds, and (b) CHON compounds.

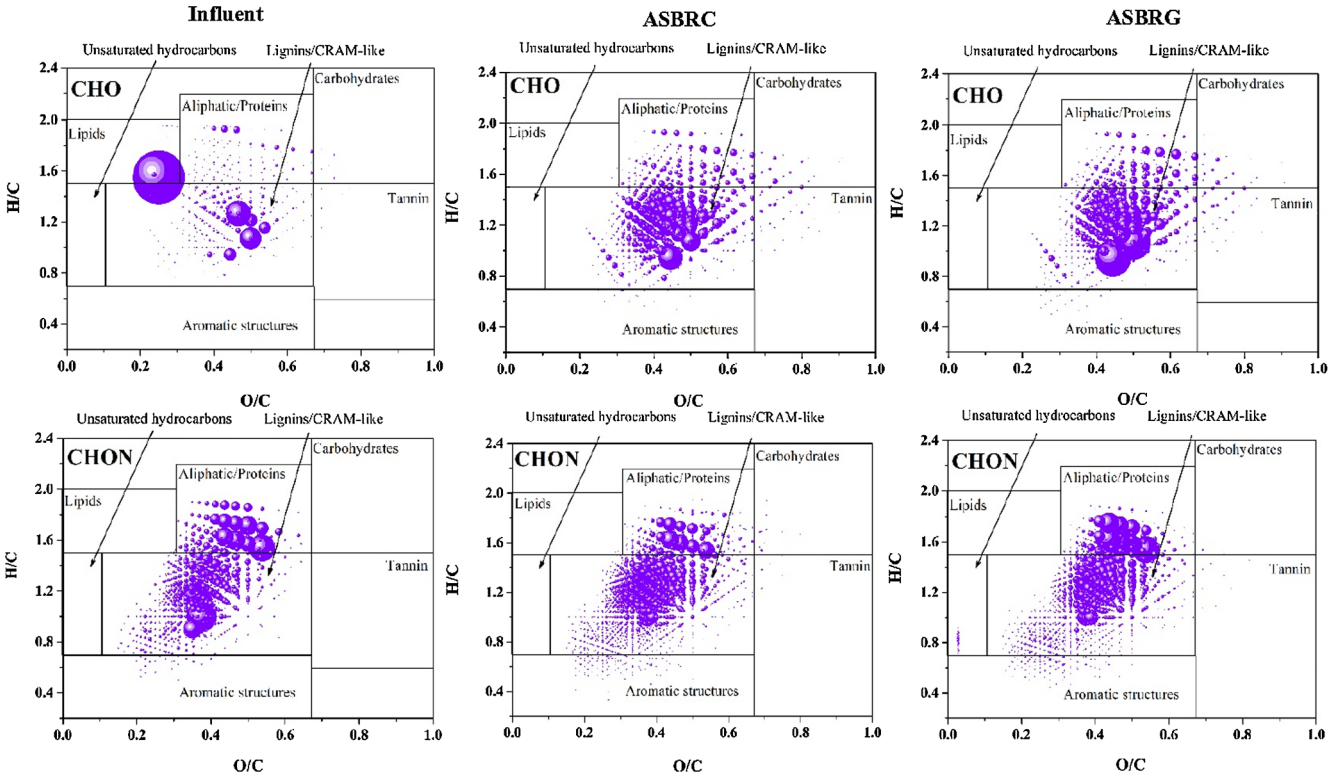


Fig. 5. Distribution of organics in HTLWW and AD effluents on the basis of major subcategories (a) CHO compounds, (b) CHON compounds.

molecules (CRAM) was in the range of 400–700 Da, which could be considered as the major refractory compounds that created the high inhibition, resulting in the formation of less methane in ASBRC (Hertkorn et al., 2006).

The more detailed class information is shown in Fig. 7. After AD, the decrease of less oxidized compounds (oxygen number < 8) in the effluent was observed. The increase of more oxidized compounds might be due to their slower degradation rate, leading to a relative increase compared with the influent. The nitrogen-containing class exhibited similar characteristics in which N1 class depleted and N2-N4 classes enriched in the effluent (Sudasinghe et al., 2014). The DBE plot (Fig. 7c) showed that lower DBE (less unsaturated) compounds were

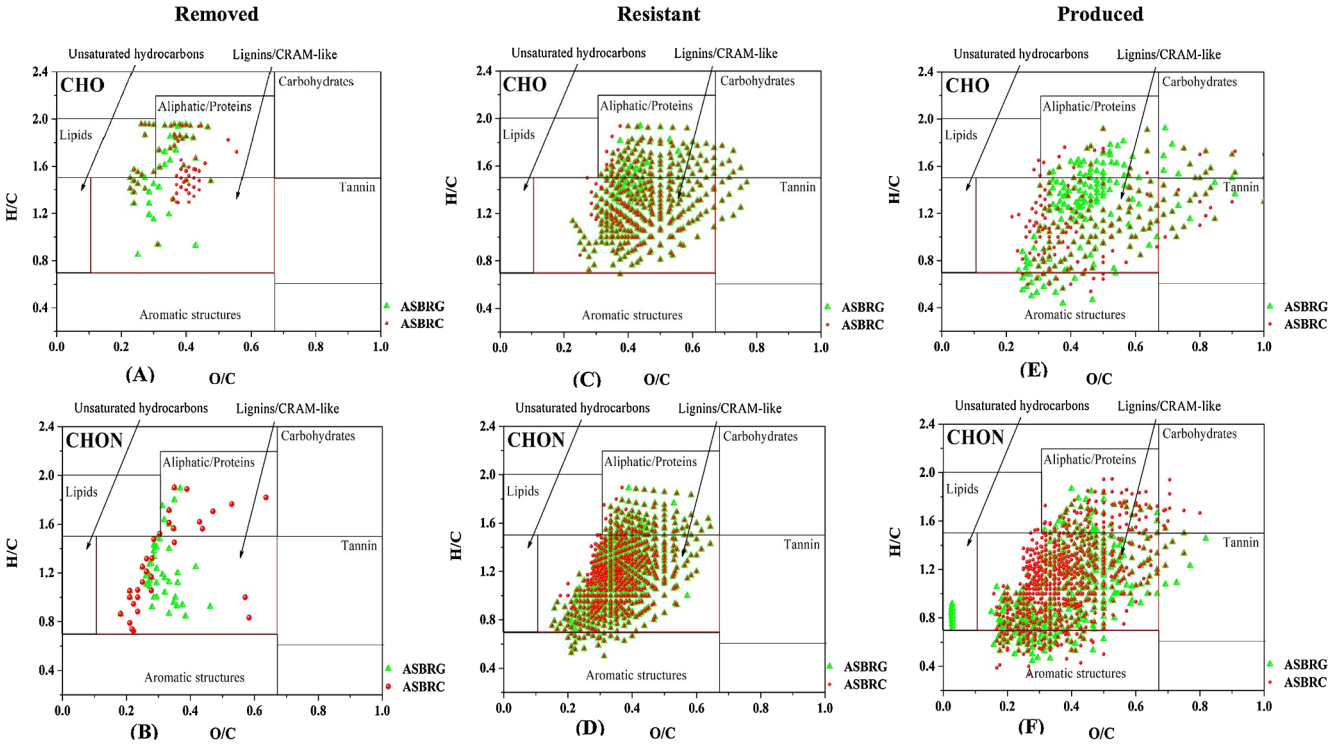


Fig. 6. Removed (A and B), resistant (C and D), and produced (E and F) organics by AD.

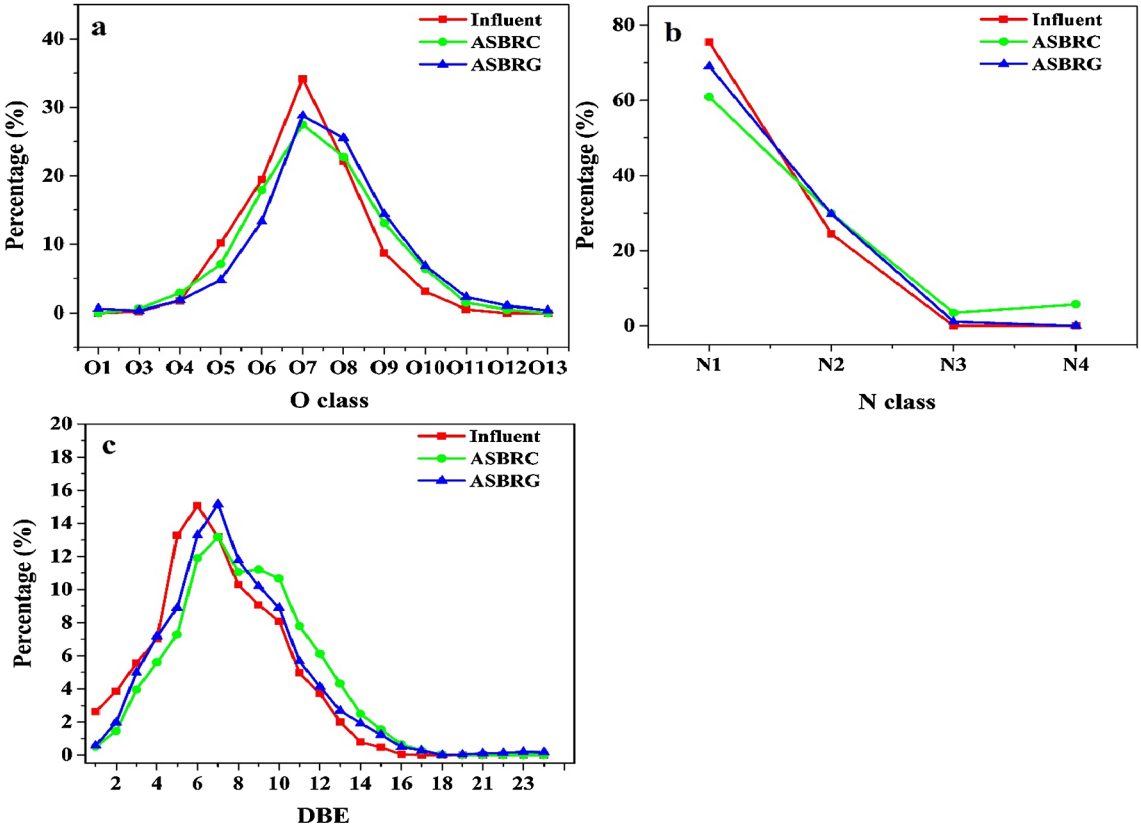


Fig. 7. Variation of CHO & CHON compounds in HTLWW and AD effluents on the basis of (a) oxygen number, (b) nitrogen number, and (c) double bond equivalent.

degraded while higher DBE compounds were produced from the residue of parent compounds. The above results demonstrated that the labile substances for anaerobic microbes were less oxidized, less unsaturated, and nitrogen-deficient compounds. The addition of GAC promoted more degradation of less oxidized compounds, molecules with high nitrogen number, and higher DBE compounds compared with control experiment. It could be due to the enrichment of certain microbes that could degrade such organics, which was also demonstrated by the following microbial analysis. All the above results showed the addition of GAC tended to affect CHON compounds more than CHO compounds. It might promote more degradation of aliphatic/protein compounds and production of unsaturated hydrocarbon.

3.5. Effect of GAC on microbial concentration and community compositions

qPCR analysis showed that the microbial concentration in ASBRG was higher than that in ASBRC (Fig. 8a), which could be due to the retention of microbes by forming biofilm on GAC. The higher microbial concentration in ASBRG was consistent with the higher methane yield. In order to better understand the role of GAC, microbial communities in the liquid samples of the two reactors and also the biofilm on GAC were further analysed by high-throughput sequencing of 16S rRNA genes. The differences of the microbial communities are shown in Fig. S7 by generating dendrograms based on Bray-Curtis similarity metrics at 0.03 cut-off. The duplicate samples were clustered together, indicating that stable microbial communities were established in the reactors. Samples $GAC_{biofilm}$ were separated from $ASBRG_{liquid}$ and $ASBRC_{liquid}$, which showed that very different microbial communities were formed in the biofilm of GAC compared with the liquid samples in the two ASBR reactors.

The results of taxonomic analysis are shown in Fig. 8b–d. At phylum level, 9 major phyla with relative abundances higher than 1% were detected from all the samples (Fig. 8b). *Firmicutes* (26.9–34.8%) was obviously enriched in $GAC_{biofilm}$ compared with $ASBRG_{liquid}$ and $ASBRC_{liquid}$. *Firmicutes* contained fermentative bacteria, which could convert the organics to VFA and then to acetic acid, H_2 and CO_2. It was reported that *Firmicutes* had the ability to degrade a wide range of organics (Zhang et al., 2018c), and its enrichment in $GAC_{biofilm}$ might be related with the degradation of more organics in HTLWW as discussed previously. Although $ASBRG_{liquid}$ and $ASBRC_{liquid}$ were clustered closer as seen in Fig. S7, there were still significant differences in the microbial communities even at phylum level. For instance, *Aminicenantes* was enriched in $ASBRG_{liquid}$ compared with $ASBRC_{liquid}$, while the relative abundance of *Chloroflexi* decreased from 15.1 to 14.3% in $ASBRC_{liquid}$ to 4.63–3.34% in $ASBRG_{liquid}$. The above results indicated that GAC addition not only specifically enriched certain microbes on its surface, but also affected the microbial communities in the liquid. It was possible that the enriched microbial community in the biofilm of GAC promoted the degradation of certain organics, and thereby resulted in the different organic compositions in the liquid, which further affected the microbial communities in $ASBRG_{liquid}$.

The bacterial genus level identification is shown in Fig. 8c. *Advenella* was dominant in $ASBRC_{liquid}$, however it was not detected in $ASBRG_{liquid}$ and $GAC_{biofilm}$. *Advenella* was isolated from human and veterinary clinical samples and had the ability to hydrolyze organics to lower molecular weight products, which might be related with the degradation of large molecular organics in HTLWW (Yue et al., 2015). The presence of GAC might promote the growth of other bacteria with similar functions. For instance, *Aminicenantes* and *Pseudomonas* were dominant in $ASBRG_{liquid}$, which could also hydrolyze various organics (Cheng and Chang, 2011; Kuroda et al., 2016). It should be noted that *Pseudomonas* was also reported to degrade aromatic compounds especially N-heterocyclic and phenolic compounds, and its enrichment in $ASBRG_{liquid}$ might be related with higher removal efficiency of COD and also nitrogenous organics as previously mentioned (Cheng and Chang, 2011; Kuroda et al., 2016; Zhong et al., 2011). Several syntrophic

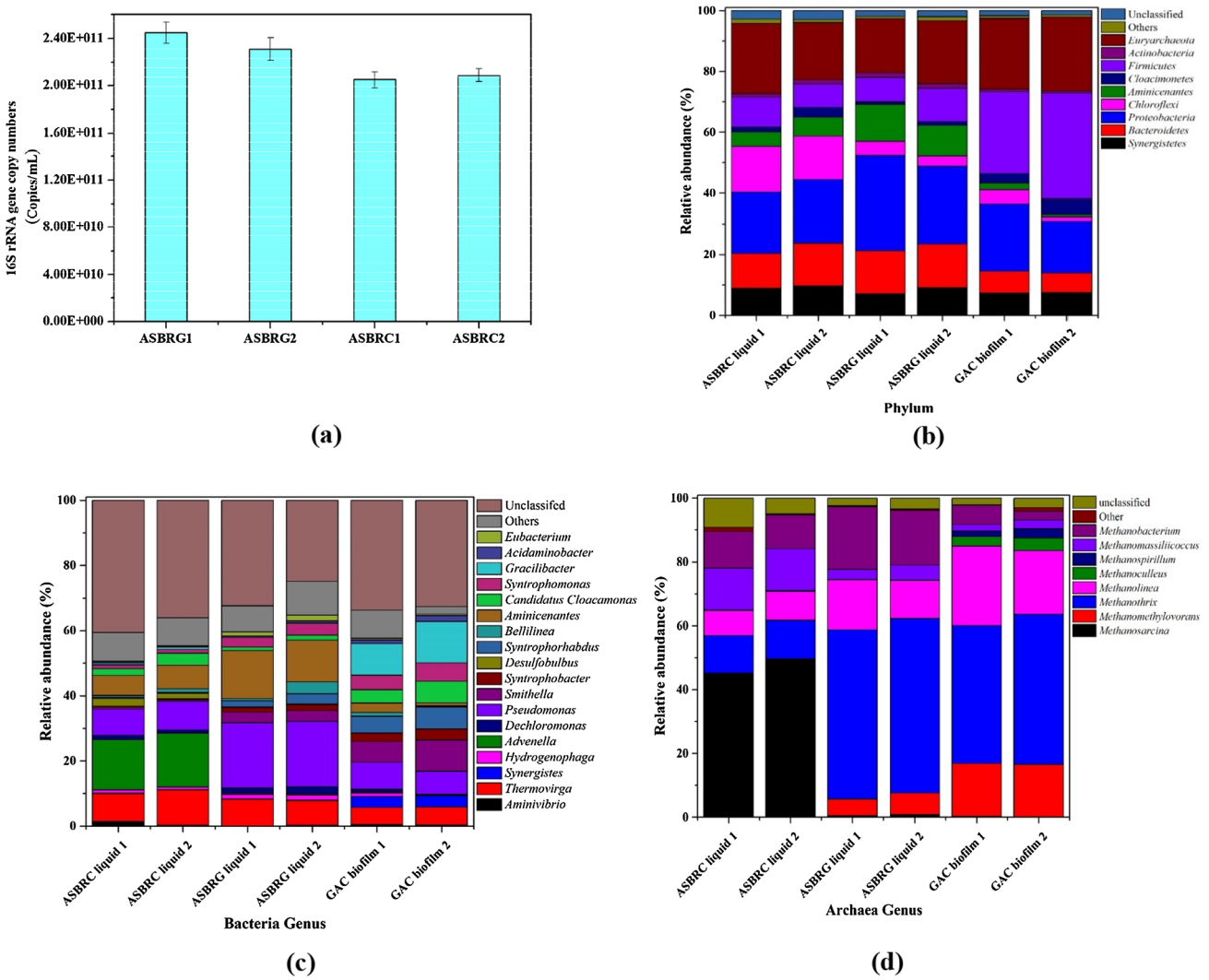


Fig. 8. Microbial concentrations (a) and variation in microbial community compositions at phylum level (b) and bacterial (c) and archaeal (d) genus level.

bacteria (*Syntrophomonas, Syntrophorhabdus, Syntrophobacter, Synergistes, Smithella*) known to perform interspecies H_2 transfer (IHT) were enriched in $GAC_{biofilm}$. *Smithella and Syntrophobacter* are syntrophic, propionate-oxidizing bacteria which utilise and remove H_2 and formate. *Syntrophomonas* utilizes the long and short chain fatty acids and has syntrophic association with hydrogenotrophic methanogens. It could also oxidize acetate and propionate by consuming protons as the electron acceptor when grown in co-culture with hydrogenotrophic methanogens (Dang et al., 2017; Hatamoto et al., 2007). *Syntrophorhabdus* is capable to degrade phenols to acetate in obligate syntrophic associations with hydrogenotrophic methanogens, and this trend was noted in GC–MS and FT-ICR-MS analysis in Table S2 and Fig. 6. *Synergistes* was found to be syntrophic acetate oxidizing bacteria in previous studies, and it was also reported to degrade nitrogenous organics (Lin et al., 2017; Si et al., 2018). The enrichment of the above syntrophic bacteria might promote the degradation of organics and thereby increased the overall COD removal efficiency. The genus *Gracilibacter* with relative abundance of 9.72–12.7% was only enriched in $GAC_{biofilm}$, and it was involved in acidogenesis stage to produce short chain fatty acids and H_2. The produced short chain fatty acids could be further utilized by the above mentioned syntrophic bacteria for acetate and H_2 production.

Fig. 8d shows the archaeal genus level identification of all the samples. *Methanosarcina* is the only known genus that could mediate both hydrogenotrophic and aceticlastic methanogenesis. It was dominant (45.2–49.5%) in $ASBRC_{liquid}$, while it was obviously decreased in the $ASBRG_{liquid}$ (0.56–0.80%) and $GAC_{biofilm}$ (0.26–0.13%). On the contrary, obvious increase of the relative abundance of *Methanothrix* (also known as *Methanosaeta*) was found in $ASBRG_{liquid}$ and $GAC_{biofilm}$ compared with $ASBRC_{liquid}$. *Methanothrix* is a strict aceteoclastic methanogens (Lin et al., 2017; Zhao et al., 2017). Previous studies showed that the dominance of either *Methanosarcina* or *Methanothrix* in AD reactors related with the concentration of acetate (Park et al., 2018; Ziganshin et al., 2011). However, the acetate concentrations were both very low in ASBRC and ASBRG in the present study. There might be other reasons affecting the dominance of the two genera. Recently, it was shown that *Methanothrix* could accept electrons via direct electron transfer (DIET) for the reduction of CO_2 to methane, and GAC could promote DIET (Lee et al., 2016; Lin et al., 2017; Park et al., 2018). Some syntrophic bacterial genus were enriched in GAC biofilm as previously mentioned, however, they mainly performed IHT and it was still not known whether they could perform DIET together with *Methanothrix* since only *Geobacter* was demonstrated for the capability to achieve DIET (Lee et al., 2016; Park et al., 2018). Based on the specific methanogenic activity analysis (Fig. S2), DIET might not be as influential as previously suggested. Nevertheless, our study showed that GAC resulted in the enrichment of *Methanothrix* in the anaerobic reactors treating HTLWW. The hydrogenotrophic methanogens *Methanolinea* and *Methanoculleus* were obviously enriched in $GAC_{biofilm}$, which could be related with the enrichment of syntrophic bacteria in

$GAC_{biofilm}$. The obligately methylotrophic genus *Methanomethylovorans* was found in both $GAC_{biofilm}$ (16.6–16.5%) and $ASBRG_{liquid}$ (5.18–6.81%), while it was not detected in $ASBRC_{liquid}$. GC–MS analysis showed that HTLWW had methyl compounds, and the enrichment of *Methanomethylovorans* indicated that GAC promoted the degradation of methyl compounds (Ziganshin et al., 2011).

3.6. Implications

Although previous studies showed that GAC were primarily used as adsorbent to remove refractory and inhibitory organics in HTLWW in order to facilitate methane production (Si et al., 2019; Usman et al., 2019; Yang et al., 2018; Zheng et al., 2017), the present study demonstrated that GAC had a long term promotion effect on methane production from HTLWW due to the increase of microbial concentration and enrichment of certain microbes. In this case, it would be more economic as GAC would not require to be regenerated. When using GAC as adsorbent before AD, the easily biodegraded organics might also be adsorbed, resulting in the less methane production. The residual organics in the effluent in the present study with the presence of GAC were mainly hard-biodegradable organics, and it can be further removed by GAC adsorption, which would reduce the amount of organics to be adsorbed by GAC. It should be noted that most recent studies thought GAC could facilitate DIET to promote methane production from various organics (Barua and Dhar, 2017; Zhao et al., 2017), the current study showed that GAC only promoted the growth of syntrophic microbes involved in IHT, which had high ability to degrade various organics. In addition, most previous studies mainly relied on microbial analysis to explain the role of GAC in AD process (Barua and Dhar, 2017; Jing et al., 2017; Zhao et al., 2017), and ignored the degradation of complex organics during this process. This could be because generally simple organics were utilized to verify the promotion effect of GAC. The present study provided an in-depth analysis of the organic degradation in HTLWW in the presence of GAC by the combination of EEM, GC–MS and FT-ICR-MS, which comprehensively characterized the organics in HTLWW. It showed that GAC enriched microbes facilitated the degradation of various organics. The microbial analysis showed that GAC mainly enriched syntrophic bacteria, and it could be due to that GAC could provide surface for the formation of biofilm, which could be suitable for the growth of certain slow growing syntrophic bacteria. Further studies should be conducted to apply GAC to different real wastewaters and to explore the possibility of GAC enriched microbes to promote the anaerobic degradation of different types of organics.

4. Conclusions

The present study showed that GAC promoted methane production from HTLWW in continuous experiments, which was attributed to the higher degradation efficiency of organics in HTLWW. The activities of both aceticlastic and hydrogenotrophic methanogens were found to be promoted by GAC. Further study showed that GAC promoted the degradation of fluorescent substances, nitrogenous and aromatic organics as revealed by EEM, GC–MS and FT-ICR-MS analysis. FT-ICR-MS analysis revealed that GAC resulted in higher removal of less oxidized compounds, molecules with high nitrogen number, and higher DBE compounds. More importantly, GAC also increased the microbial concentration and had significant effects on the microbial community compositions. More syntrophic bacteria with the ability to degrade various organics were enriched, and the dominance of *Methanosarcina* in methanogens was replaced by *Methanothrix* in the presence of GAC.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105257.

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