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Resistance of anaerobic activated sludge acclimated by different feeding patterns: response to different stress shocks

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ABSTRACT

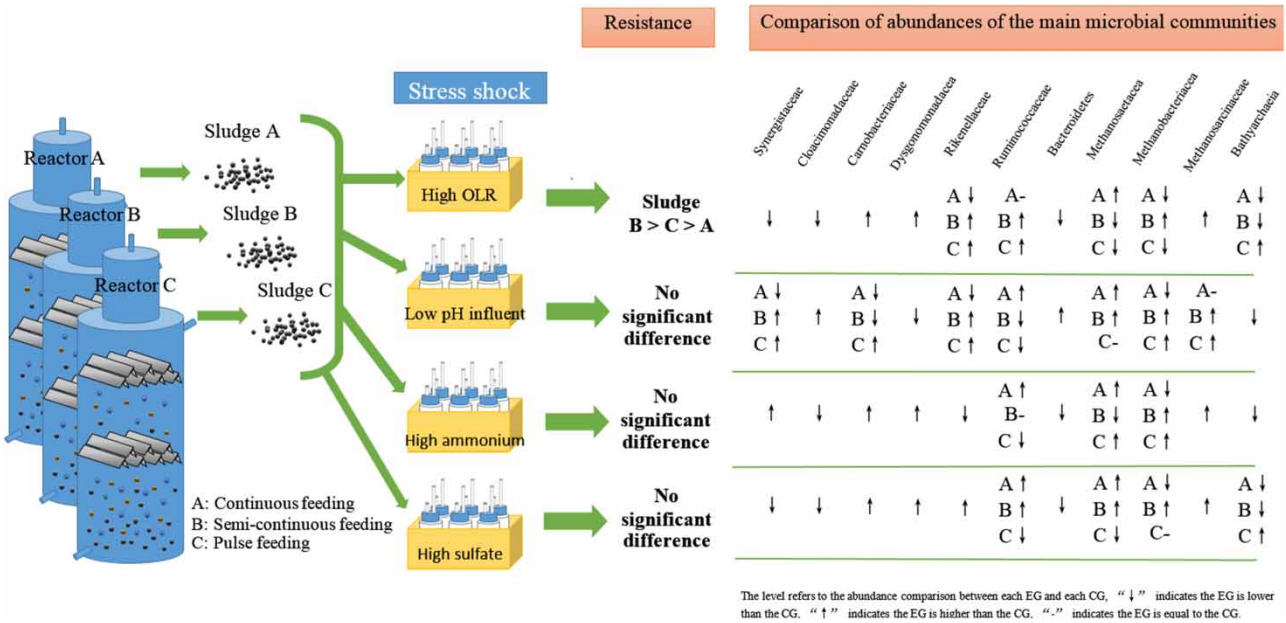
Anaerobic activated sludge plays a key role in the anaerobic digestion (AD) treatment of wastewater. The ability of anaerobic activated sludge to endure stress shock determines the performance of AD. In this study, the resistance of anaerobic activated sludge acclimated by three feeding patterns (continuous, semi-continuous, and pulse) to four stress shocks, including low pH influent, high OLR (organic loading rate), high ammonium and high sulfate, was investigated respectively. The results showed that the anaerobic activated sludge acclimated by semi-continuous feeding had the best resistance to high OLR shock, followed by pulse feeding, and then continuous feeding. There was no significant difference in the resistance of the three activated sludge to the other stress shocks. Under stress shock, the microbial community structure and abundance of specific functional microorganisms in the activated sludge acclimated by different feeding patterns varied, while the relative abundance of *Methanosarcinaceae* in the anaerobic activated sludge increased. The variation in the relative abundance of specific functional microorganisms was in charge of the differences in the resistance of anaerobic activated sludge. Overall, the results presented herein provide reference for improving the stability and effectiveness of activated sludge under adverse conditions.

Key words: anaerobic activated sludge, feeding pattern, microbial community, resistance, stress shock

HIGHLIGHTS

- Resistance of activated sludge acclimated by three feeding patterns was studied.
- Acclimated activated sludge was well resistant to low pH influent and high sulfate.
- Sludge acclimated by semi-continuous feeding can best resist to high OLR shock.
- The relative abundance of Methanosarcinaceae increased under stress shock.
- Differences in functional microbial abundance led to different resistance of sludge.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Molasses alcohol wastewater (MAW) is a high-strength organic wastewater generated in the process of producing alcohol from molasses. This wastewater is characterized by high chemical oxygen demand (COD; 80–160 g/L), high levels of dissolved inorganic matter, low pH (3.7–4.5) and a dark color, which bring great challenges to its disposal (Basu *et al.* 2015). Anaerobic digestion (AD) is an efficient technology that is widely accepted for wastewater treatment. Several studies on the effective treatment of industrial wastewater containing MAW using AD have been conducted (Zhang *et al.* 2011; Shen *et al.* 2014; Gagliano *et al.* 2017; Granada *et al.* 2018). However, the varied characteristics of the wastewater because of raw materials and operating conditions result in the instability of the treatment system.

The anaerobic activated sludge plays a key role in the AD treatment of wastewater, and its performance determines the efficiency of AD. Because of fluctuations in the characteristics of industrial wastewater, the anaerobic activated sludge process is subjected to stress shocks, while usually consist of changes in pH, temperature, organic load, hydraulic load, ammonia nitrogen (NH_4^+-N) or salinity (Zandvoort *et al.* 2005; Sabry 2008; Gao *et al.* 2011; Li *et al.* 2014; Zhang *et al.* 2017, 2018; Wang *et al.* 2018). These shocks, combined with drawbacks of AD, such as unstable operation, poor resistance to high-load variations and transient operation conditions (Couras *et al.* 2014), are likely to have serious impacts on the AD system, and may even cause the system to collapse.

During anaerobic treatment of MAW, the system is most likely to be subjected to the stress shock of low pH, high organic load, high concentrations of NH_4^+-N and high sulfate. Low pH, high organic load and high sulfate stress shock occur because of the characteristics of MAW. Sulfuric acid is used to regulate the pH in the molasses alcohol production process, which results in high sulfate content in MAW. The sulfate concentration in MAW is usually higher than 6,000 mg/L. Although the concentration of NH_4^+-N in MAW is low, the nitrogen in the wastewater will be converted into ammonium nitrogen in the anaerobic treatment process, resulting in increasing the concentration of NH_4^+-N in the anaerobic reactor. In a well-run anaerobic reactor for treating MAW, the NH_4^+-N concentration can be above 1,000 mg/L, while it is higher in poorly run reactors.

Variation in pH has been reported to directly influence activated sludge organisms in anaerobic reactors (Chong *et al.* 1997). Changes in pH mainly influence microbial metabolism, including the utilization of substrates, synthesis of proteins and various types of storage materials, as well as release of metabolic products from cells (Gao *et al.* 2010). Regulation of the organic loading rate (OLR) is the key operational unit in anaerobic wastewater treatment. Changes in the OLR cause variations in the ratios of substrates to microorganisms and dissolved oxygen levels in the anaerobic reactor, which affects the

utilization efficiency of substrates by microorganisms. A high OLR may lead to unbalanced kinetic rates in the pathways of AD, resulting in methane yield reduction and volatile fatty acids (VFA) accumulation (Polizzi *et al.* 2018). High-level NH_4^+-N would inhibit the activity of microorganisms in anaerobic reactors and seriously affect the efficiency of the reactor (Yang *et al.* 2018a, 2018b). The mechanism of NH_4^+-N inhibition is universally acknowledged that high levels of ammonia change the intracellular pH, increase the required maintenance energy, and deplete intracellular potassium, and inhibit specific enzyme reactions (Gao *et al.* 2015). During the anaerobic treatment of wastewater, the presence of sulfate leads to decreased methane production and produces malodorant H_2S, which is toxic to microorganisms. This occurs because sulfate stimulates the growth of sulfate reducing bacteria (SRB) and competes with methanogens for hydrogen and acetate substrates in the reactor, inhibiting the growth of methanogens. H_2S produced in the AD process readily permeates the cell membrane and produces sulfide and disulfide cross-links between polypeptide chains inside the cytoplasm to denature native proteins (Siles *et al.* 2010).

Previous studies have shown that the feeding pattern influences the settling characteristics (Caluwé *et al.* 2017) and the microbial community structure of anaerobic activated sludge (Park *et al.* 2018; Lu *et al.* 2019). However, performance and resistance of activated sludge acclimated by different feeding patterns subjected to stress shock have not yet been investigated. To date, several studies have investigated the impact of the anaerobic activated sludge on stress shock, such as the temperature, pH and OLR, but few have investigated ammonia and sulfate shock. Moreover, no studies comparing the resistance of activated sludge acclimated using different feeding patterns to pH, OLR, high ammonium or high sulfate shock have been published. In view of the vital role of anaerobic activated sludge in the AD process, more studies on stress resistance of anaerobic activated sludge are needed.

Therefore, this study was conducted to investigate the resistance of anaerobic activated sludge acclimated by three feeding patterns (continuous, semi-continuous, and pulse) to four stress shocks respectively, including low pH influent, high OLR, high ammonium and high sulfate. Whether there were differences in the resistance of activated sludge acclimated by three feeding patterns to four stress shocks and the relationship between the resistance and the microbial community of activated sludge were detected. To accomplish this, key parameters, such as the soluble chemical oxygen demand (SCOD) removal rate, pH of effluent, VFA and NH_4^+-N concentration was monitored. In addition, variations in the microbial community structure were investigated.

2. MATERIALS AND METHODS

2.1. Experimental set-up and operation

Eighteen anaerobic lab-scale reactors, each with a total volume of 500 mL and a working volume of 400 mL, were operated at 35 °C, and a hydraulic retention time (HRT) for 4 days. The reactors consisted of three groups of six. The six reactors in each group were divided into a control group (CG) and an experimental group (EG), with three reactors in each group considered biological replicates. The three groups of reactors were inoculated with three kinds of activated sludge that originated from three AD reactors. The three AD reactors had treated MAW for 148 d by continuous, semi-continuous and pulse feeding pattern respectively, and denoted A, B and C, respectively (Table 1). Three feeding patterns were operated by peristaltic pump with controlled time and flow. Sludge A, B, and C was derived from the corresponding A, B, and C reactors respectively. Sludges A, B and C were diluted with tap water to 1 kg/L by weight, and then inoculated into the AD systems, which were subsequently used to investigate the resistance of the three sludges to stress shocks, including low pH influent, high OLR, high ammonium and high sulfate (Table 2). The pH of influent was adjusted with NaOH. All of the systems were fed daily with 6 g SCOD L^{-1} d^{-1}, with the exception of the high OLR treatment, in which the OLR was as shown in Table 2. Ammonium and sulfate were applied as NH_4Cl and Na_2SO_4, respectively. At the start of the experiment, each reactor

Table 1 | Feeding and operational pattern for each sludge

Feeding pattern	Operational pattern	Feeding time (h)	Sludge
Continuous feeding	24 L/3 d	72	A
Semi-continuous feeding	24 L (Day 1), 0 L (Days 2 and 3)	24	B
Pulse feeding	8 L/once/d, total 24 L/3 d	12 (4 h/once)	C

Table 2 | Stress tests set-up

Stressors	Day 1	Days 2–5	Days 6–9	Days 10–13	Days 14–17	Day 18–21	Days 21–25
Control	–	–	–	–	–	–	–
pH of influent	6.0	5.5	5.0	4.5	3.8	3.8	3.8
OLR (g SCOD/L/d)[a]	6	8	10	12	14	16	
Ammonium (mg TAN/L)	0	500	1,000	2,000	3,000		
Sulfate (mg/L)	0	500	1,000	2,000	3,000		

[a]In every treatment, except for the high OLR treatment, the OLR was 6 g SCOD L^{-1} day^{-1}. The values presented for ammonium and sulfate are final concentrations in the reactor ($n = 3$). OLR (organic loading rate), SCOD (soluble chemical oxygen demand), TAN (total ammonia nitrogen).

was filled with 100 mL activated sludge and 300 mL MAW (OLR = 6 g SCOD L^{-1} d^{-1}). All of the reactors were placed in a water bath to maintain a temperature of 35 °C. 100 mL MAW was injected and 100 mL effluent was collected in each reactor using syringes daily. The pH of the effluent was monitored daily, while the SCOD removal rate, concentration of NH_4^+-N and VFAs of the effluent was measured every four days.

2.2. Analytical procedures

The SCOD and NH_4^+-N was determined according to Standard Methods for Water and Wastewater Monitoring and Analysis Methods published by China Environmental Science Press. The pH was monitored using a pH meter. The concentration of VFA was determined using a gas chromatograph (Shimadzu, GC-2010 Plus, DB-FFAP column) equipped with a flame ionization detector. The testing procedure: 70 °C 1 min, the temperature was increased to 180 °C /min at 20 °C /min and kept for 3 min, and then continue to increase the temperature at 20 °C /min to 200 °C for 3 min.

2.3. Microbial community analyses

Activated sludge samples of CGs and EGs after the stress shock test were collected for microbial community analysis. Total DNA was extracted from the activated sludge samples using a FastDNA Spin Kit for Soil (MP Biomedicals). Partial sequences of the 16S rRNA gene including the variable V3–V4 region were amplified from the total DNA using the following primers: 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) for bacteria, Arch349F (5′-GYGCASCAGKCGMGAAW-3′) and Arch806R (5′-GGACTACVSGGGTATCTAAT-3′) for methanogens. Sequencing methods of PCR products and the data processing and analysis methods were consistent with the references (Lu *et al.* 2019).

3. RESULTS AND DISCUSSION

3.1. Resistance of activated sludge to low pH influent

The anaerobic activated sludge A, B and C acclimated by different feeding patterns was subjected to continuous low pH influent shock for 25 consecutive days, after which the performance of the CGs and EGs was measured. The results were shown in Table 3. On day 25, the effluent pH of the CG AD systems was about 7.60, while that of the EG AD systems was about 7.50. The SCOD removal rate for all of the AD systems was about 65.0%. The NH_4^+-N concentrations of the A, B and C AD systems were between 760 mg/L and 840 mg/L. The VFA concentrations of all the AD systems were below 600 mg/L. The overall level of VFAs was relatively low for all systems. Additionally, the VFA components in CGs and EGs were primarily acetic acid and propionic acid, while the other components were almost undetectable. The results showed that low pH influent shock had little effect on the effluent pH, SCOD removal rate, NH_4^+-N and VFA concentration of AD systems treating A, B and C, indicating these sludges has favorable resistance to low pH influent that did not differ significantly. Gao *et al.* (2010) found that extreme pH shock induced the dispersion of sludge flocs and accumulating colloids and solutes or biopolymers in the sludge suspension of a submerged anaerobic membrane bioreactor and deteriorated the performance of the bioreactor. In the present study, sludges A, B and C were all acclimated by MAW for a long time, and had a strong capacity to buffer pH. Therefore, when only the pH of the influent decreased, the AD systems could self-regulate and recover quickly; accordingly, pH influent shock had little influence on the performance of AD systems.

Table 3 | The performance of activated sludge anaerobic digestion systems under stress shock

Parameter \ Stressor		Low pH influent		High OLR		High ammonium		High sulfate	
		CG	EG	CG	EG	CG	EG	CG	EG
Effluent pH	A	7.60 ± 0.01	7.48 ± 0.02	7.57 ± 0.01	7.50 ± 0.06	7.35 ± 0.01	6.61 ± 0.03	7.35 ± 0.01	7.49 ± 0.02
	B	7.59 ± 0.05	7.48 ± 0.02	7.54 ± 0.0	7.63 ± 0.02	7.30 ± 0.02	6.55 ± 0.02	7.32 ± 0.01	7.47 ± 0.02
	C	7.63 ± 0.01	7.46 ± 0.03	7.57 ± 0.0	7.51 ± 0.01	7.31 ± 0.01	6.59 ± 0.05	7.33 ± 0.01	7.48 ± 0.01
SCOD removal rate (%)	A	65.5 ± 2.1	68.0 ± 0.2	67.9 ± 0.4	41.9 ± 3.3	74.6 ± 1.3	36.2 ± 1.5	71.2 ± 1.0	65.0 ± 1.0
	B	66.6 ± 2.1	65.3 ± 0.6	66.4 ± 1.4	49.0 ± 1.9	74.2 ± 0.8	36.4 ± 1.4	70.2 ± 0.6	61.5 ± 1.0
	C	65.5 ± 0.7	65.7 ± 1.5	65.0 ± 0.6	41.1 ± 0.3	74.6 ± 0.2	37.4 ± 0.9	70.5 ± 0.5	61.8 ± 0.6
Ammonia nitrogen (mg/L)	A	800 ± 20	800 ± 25	680 ± 10	2,000 ± 40	480 ± 10	11,400 ± 240	520 ± 10	480 ± 10
	B	840 ± 15	880 ± 20	800 ± 20	1,900 ± 45	440 ± 10	11,700 ± 260	520 ± 10	480 ± 10
	C	760 ± 20	760 ± 15	720 ± 25	1,850 ± 35	480 ± 10	11,700 ± 230	520 ± 15	480 ± 10
Total VFA (mg/L)	A	544 ± 34	209 ± 29	1,530 ± 83	13,790 ± 37	399 ± 36	3,337 ± 25	430 ± 59	564 ± 30
	B	445 ± 38	544 ± 37	2,062 ± 57	9,657 ± 49	535 ± 20	3,993 ± 22	414 ± 26	858 ± 33
	C	462 ± 42	367 ± 37	1,851 ± 79	13,915 ± 43	528 ± 7.7	4,164 ± 32.7	458 ± 27	843 ± 28

3.2. Resistance of activated sludge to high OLR

Sludges A, B and C were subjected to increasing concentrations of high OLR shock for 21 consecutive days, during which time the performance of the CG and EG AD systems was measured. With the increase of OLR, SCOD removal rate of the EG AD systems decreased, while NH_4^+-N and VFA concentrations increased. As shown in Table 3, on day 21, the effluent pH of the CG and EG AD systems was between 7.50 and 7.63. Additionally, SCOD removal rates of EGs A, B and C were 26.0, 17.4 and 23.9% lower, respectively, than those of the CGs on day 21. On day 21, the NH_4^+-N concentration of the EG A, B and C increased by 194, 138 and 157%, while the VFA concentration increased by 801, 367 and 652%, respectively, compared with the respective CGs. The NH_4^+-N and VFA concentration of the EG A, B and C was much higher than that of the respective CGs, indicating that the performance of the EG AD systems was seriously affected. Moreover, EG B AD system was the least affected, followed by EG C, and EG A was the most affected. The main VFA components in the CGs were acetic acid and propionic acid, while the other components were almost undetectable. However, in all of the EGs, the concentrations of isobutyric acid, n-butyric acid, isovaleric acid and n-pentanoic acid increased, while the distribution of acetic acid declined. A previous study suggested that increasing the OLR changes the VFAs type from acetic acid to n-butyric acid (Wijekoon *et al.* 2011). These results showed that a high OLR had a significant effect on the performance of A, B and C AD systems and severely inhibited the digestion efficiency. Furthermore, there were significant differences among groups A, B and C. The SCOD removal rate of EG B decreased the least compared with the respective CG, followed by that of C and then A. The NH_4^+-N and VFA concentration of EG B increased the least compared with the respective CG, followed by that of C and then A. These findings indicated that activated sludge B had the best resistance to high OLR, followed by C and finally A. The difference in resistance of the three activated sludges to high OLR may be related to the methods used for their domestication. Specifically, during the domestication of activated sludge, the fluctuations in their OLR were different. As shown in Table 1, sludge A was acclimated by a continuous feeding pattern, with the flow of influent kept stable; however the flow of influent changed for sludges B and C. As a result, the effects of fluctuations of OLR on activated sludges B and C were more severe than on activated sludge A.

3.3. Resistance of activated sludge to high ammonium

The anaerobic activated sludges A, B and C were subjected to increasing concentrations of ammonium shock for 17 consecutive days, during which time the performance of the corresponding CGs and EGs was measured. As shown in Table 3, with the increase in ammonium concentration, SCOD removal rate of the EG AD systems decreased, while ammonia nitrogen and VFA concentrations increased. On day 17, the effluent pH of CGs A, B and C AD systems was 7.35, 7.30 and 7.31, while that of the EGs was 6.61, 6.55 and 6.59, respectively. The effluent pH of each EG was lower than that of the CG, but there were no significant differences among groups. SCOD removal rates of EGs A, B and C were 38.4, 37.8 and 37.2% lower than those of the CGs, respectively, on day17. On day 17, the NH_4^+-N concentration of EGs A, B and C increased by 2,275, 2,559 and 2,338% compared with the respective CGs, while VFA concentrations of EGs A, B and C increased by 736, 646 and

689%. The NH_4^+-N and VFA concentration of the EG A, B and C was much higher than that of the respective CGs, indicating that the performance of the EG AD systems was seriously affected, or even collapsed. There was no significant difference in the performance of each system. The VFA components in the CGs were acetic acid and propionic acid, while the other components were almost undetectable. However, in all of the EGs, the concentration of isobutyric acid, n-butyric acid and isovaleric acid increased, while the distribution of acetic acid declined. Acetic acid was the most conducive to methanogenesis, and the contribution of acetic acid to methanogenesis is more than 70% (Wijekoon *et al.* 2011). Other types of VFA need to be transformed into acetic acid through microbial metabolism. The accumulation of other types of VFA, especially propionic acid, can easily lead to acidification and even collapse of the reactor. The decrease of acetic acid and the increase of other types of VFA in all of the EGs meant that the performance was affected. A previous study reported that excessive NH_4^+-N concentrations (e.g., 1,460–5,620 mg/L) could severely inhibit microbial communities involved in AD (Buhlmann *et al.* 2019). In this study, the NH_4^+-N concentrations of EGs were over 10,000 mg/L at day 17, resulting in significant decreases in the efficiency of the three EG AD systems. Specifically, the SCOD removal rates all decreased to less than 40% and the VFA concentrations increased by over 2,500 mg/L. These findings were in agreement with those of a previous report, showing that ammonia inhibition of AD system led to decreased efficiency and accumulation of VFAs (Rajagopal *et al.* 2013). Although high ammonium shock had a significant effect on the three activated sludge anaerobic systems, there was no difference in the resistance of the three activated sludges to NH_4^+-N. Angelidaki & Ahring (1993) reported that NH_4^+-N tolerance could increase through an adapted process. The concentrations of NH_4^+-N in anaerobic reactors with different feeding patterns differed, which may lead to different adaptability of activated sludge to NH_4^+-N in the reactors. However, because the NH_4^+-N concentration of influent(about 52 mg/L) was far lower than the concentrations of NH_4^+-N to induce shock (500–3,000 mg/L), the resistance of three activated sludge to NH_4^+-N shock showed no difference.

3.4. Resistance of activated sludge to high sulfate

Sludges A, B and C were subjected to increasing concentrations of sulfate shock for 17 consecutive days, during which time the performance of the corresponding CGs and EGs was measured. As shown in Table 3, on day 17, the effluent pH of the A, B and C CGs and EGs ranged from 7.30 to 7.50, while the NH_4^+-N concentrations ranged from 480 to 520 mg/L, with no significant differences among groups. SCOD removal rates of the A, B and C EGs were 6.2, 8.7 and 8.7% lower than those of the CGs, respectively, on day17. On day 17, VFA concentrations of EGs A, B and C increased by 31, 107 and 84%, respectively, when compared with the respective CG. The VFA concentration of the EG A, B and C was slightly higher than that of the respective CGs, but the concentration was within the normal range and had little influence on the performance of AD system. VFA components in CGs and EGs were primarily acetic acid and propionic acid, while the other components were almost undetectable. Jing *et al.* (2013) reported that SRB would compete with methanogens for substrates such as acetate and hydrogen in the presence of sulfate. SRB often outcompete methanogens and produce toxic sulfide during reduction. The accumulation of sulfide causes a toxic effect on SRB and methanogens, which leads to inhibition of organics removal and methane production, and can even lead to failure of the AD. The total organic carbon (TOC) removal efficiency and biogas production rate decreased slightly in response to the addition of 500–800 mg/L sulfate in an anaerobic biofilm reactor (Yang *et al.* 2015). Moreover, Bernardez *et al.* (2012) showed that initial sulfate concentration of 1,000 and 3,500 mg/L inhibited anaerobic reactor performance. In the present study, the maximum amount of sulfate added in the EGs was 3,000 mg/L. When compared with the CGs without added sulfate, the SCOD removal rate decreased slightly, the VFA content was slightly higher and the pH and NH_4^+-N levels of the effluent were not different in the EGs. These results indicated that the addition of sulfate inhibited the EGs AD systems, but the inhibition was not severe. Acclimated activated sludge was also demonstrated to have resistance to sulfate.

3.5. Microbial community analysis

3.5.1. Bacterial community analysis

The bacterial community of AD systems A, B and C under stress shock was investigated based on the composition and structure of bacterial 16S rRNA genes obtained by high-throughput multiplex sequence analysis. The distribution of the main bacterial community abundance at the family level is shown in Figure 1. When the AD systems of activated sludge were subjected to low pH influent shock, the abundance of *Cloacimonadaceae* in EG A, B and C was 1.68, 3.56 and 6.81% higher than that in each CG, while the abundance of *Dysgonomonadaceae*, *Endomicrobiaceae* and *Anaerolineaceae* in each EG was lower than in CG. When the AD systems of the activated sludge were subjected to high OLR shock, the abundance of

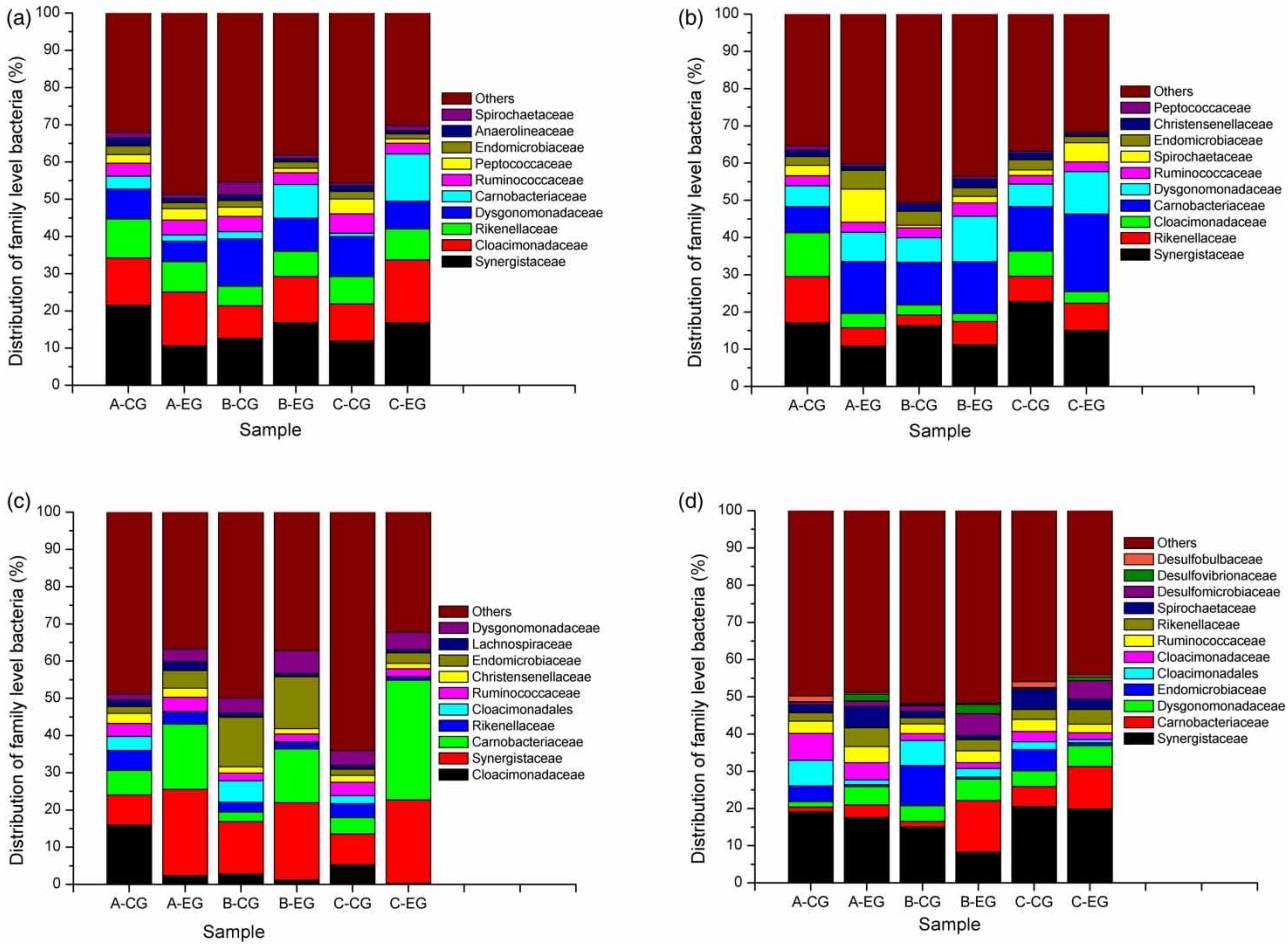


Figure 1 | Distribution of the main bacterial community abundance at the family level. (a) Low pH influent shock, (b) high OLR shock, (c) high ammonium shock, and (d) high sulfate shock.

Carnobacteriaceae in EG A, B and C was 6.89, 2.45 and 8.97% higher than in each CG, the abundance of *Dysgonomonadaceae* in EG A, B and C was 2.21, 5.68 and 5.29% higher than in each CG, while the abundance of *Synergistaceae* and *Cloacimonadaceae* in each EG was lower than in each CG. When the AD systems of activated sludge were subjected to high ammonium shock, the abundance of *Synergistaceae*, *Carnobacteriaceae* and *Dysgonomonadaceae* in each EG was higher than in each CG, while the abundance of *Cloacimonadaceae*, *Rikenellaceae*, *Cloacimonadales* and *Christensenellaceae* in each EG was lower than in each CG. When the AD systems of the activated sludge were subjected to high sulfate shock, the abundance of *Dysgonomonadaceae*, *Rikenellaceae*, *Desulfomicrobiaceae* and *Desulfovibrionaceae* in each EG was higher than in each CG, while the abundance of *Synergistaceae*, *Endomicrobiaceae*, *Cloacimonadales* and *Cloacimonadaceae* in each EG was lower than in each CG.

The results of bacterial community principal component analysis (PCA) in activated sludge of AD systems are presented in Figure 2. The bacterial community of activated sludge was significantly affected by stress shock. The bacterial community of activated sludge acclimated by different feeding patterns was affected differently. When the AD systems of activated sludge were subjected to high OLR shock, the minimum difference of bacterial community was sludge B, followed by sludge C, and the maximum difference was sludge A between the CGs and EGs.

AD is a complex and continuous process of degrading organic matter that is accomplished by the collaboration of three distinct groups of microorganisms. These groups of microorganisms include acidogenic bacteria, acetogenic bacteria and methanogenic archaea. In a normal AD system, these microorganisms coordinate and keep well balanced to promote system stability and good performance; however they are sensitive to disturbances. Although Allison & Martiny (2008) have reported that microbial communities were resistant and resilient to disturbances, this necessary equilibrium would be

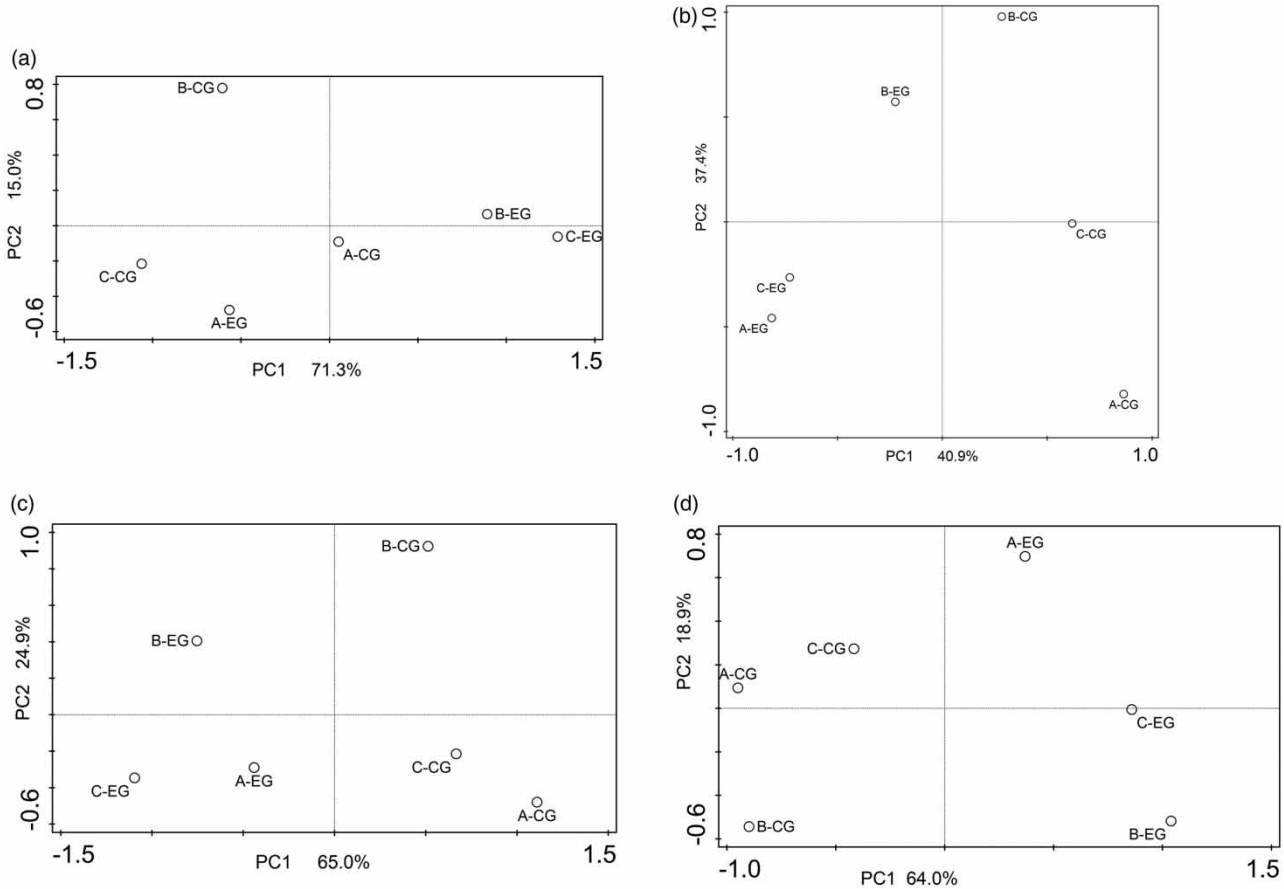


Figure 2 | Principal component analysis based on bacterial community. (a) Low pH influent shock, (b) high OLR shock, (c) high ammonium shock, and (d) high sulfate shock.

altered when the impact of the disturbance exceeds the self-regulatory ability of microbial communities. When the AD system was subjected to stress shock, the microbial communities shifted. In this present study, the main bacterial communities in all of the anaerobic activated sludge samples were *Synergistaceae*, *Cloacimonadaceae*, *Carnobacteriaceae*, *Dysgonomonadac*, *Rikenellaceae* and *Ruminococcaceae*. When being subjected to high OLR, high ammonium and high sulfate shock, the relative abundance of *Cloacimonadaceae* in the activated sludge of EGs decreased, while the relative abundance of *Carnobacteriaceae* and *Dysgonomonadaceae* increased compared with each CG. *Cloacimonadaceae* has been reported to be related to fatty acid degradation (Dyksma & Gallert 2019; Shakeri Yekta *et al.* 2019). *Carnobacteriaceae* has been reported to be involved in the production of VFA and its abundance was shown to increase in response to high ammonium levels (Regueiro *et al.* 2016). Whon *et al.* (2015) reported that *Carnobacteriaceae* contains gene clusters involved in the production of lactate and butyrate. Therefore, the decreased abundance of *Cloacimonadaceae* indicates that the conversion efficiency of VFA was reduced, while increased abundance of *Carnobacteriaceae* indicates the production of more VFAs during AD. Changes in abundance of *Cloacimonadaceae* and *Carnobacteriaceae* led to the accumulation of VFAs in the AD system, which was consistent with the significant increase of total VFAs and butyric acid in EGs described in sections 3.2, 3.3 and 3.4. A previous study reported that *Dysgonomonadaceae* played an important role in hydrolysis and fermentation, and was capable of degrading various polysaccharides (Murakami *et al.* 2018; Wang *et al.* 2019a). The increased abundance of *Dysgonomonadaceae* may be because of an increase of hydrolytic and fermentative substrates, which need to be quickly converted into small molecules for use by other microbial communities to maintain the stability of the anaerobic digestive system. When subjected to high OLR and high sulfate shock, the relative abundance of *Synergistaceae* in the activated sludge of EGs decreased, but the relative abundance increased in response to high ammonium shock. Ziganshina *et al.* (2015) reported that the relative abundance of *Synergistaceae* decreased when total NH_4^+-N, VFAs and pH in the AD reactor increased. As shown

in sections 3.2, 3.3 and 3.4, the NH_4^+-N and VFAs of EGs increased to varying degrees when subjected to high OLR, high ammonium and high sulfate shocks, while the pH of EGs remained above 7.30 under high OLR and high sulfate shocks, and the pH of EGs dropped below 6.61 under high ammonium shock. The decreased pH may be responsible for the increased relative abundance of *Synergistaceae* in the EGs under high ammonium shock. *Synergistaceae* are involved in VFA transformation (Suksong *et al.* 2019). Ma *et al.* (2019) reported that *Synergistaceae* could decompose organic acids into acetate with the production of H_2 in co-culture with H_2-consuming methanogens, resulting in improvements in hydrolysis acidification and the acetotrophic pathway. The relative abundance of *Rikenellaceae* and *Ruminococcaceae* in the activated sludge of EG B and C increased compared with each CG, but was decreased or constant in EG A under high OLR shock. Correlation analysis indicated that *Rikenellaceae* and *Ruminococcaceae* had the potential to degrade polysaccharides (Tao *et al.* 2019). Notably, the relative abundance of *Desulfomicrobiaceae* and *Desulfovibrionaceae* in each EG was higher than in each CG (Figure 3(d)). *Desulfomicrobiaceae* and *Desulfovibrionaceae* are SRB, and previous investigations of SBR have shown that the presence of sulfate stimulates SRB growth (Zhang *et al.* 2013). *Desulfovibrionaceae*, which is the main group of SRB, can utilize VFA as an electron donor to reduce sulfate; however, SRB compete with methanogens for substrates and produce toxic sulfide, inhibiting methanogens.

3.5.2. Archaeal community analysis

The archaeal communities of AD system of A, B and C under stress shock were investigated by comparing the composition and structure of 16S rRNA genes obtained by high-throughput multiplex sequence analysis. The distribution of the main archaeal community abundance at the family level is shown in Figure 3 When the AD systems of activated sludge were

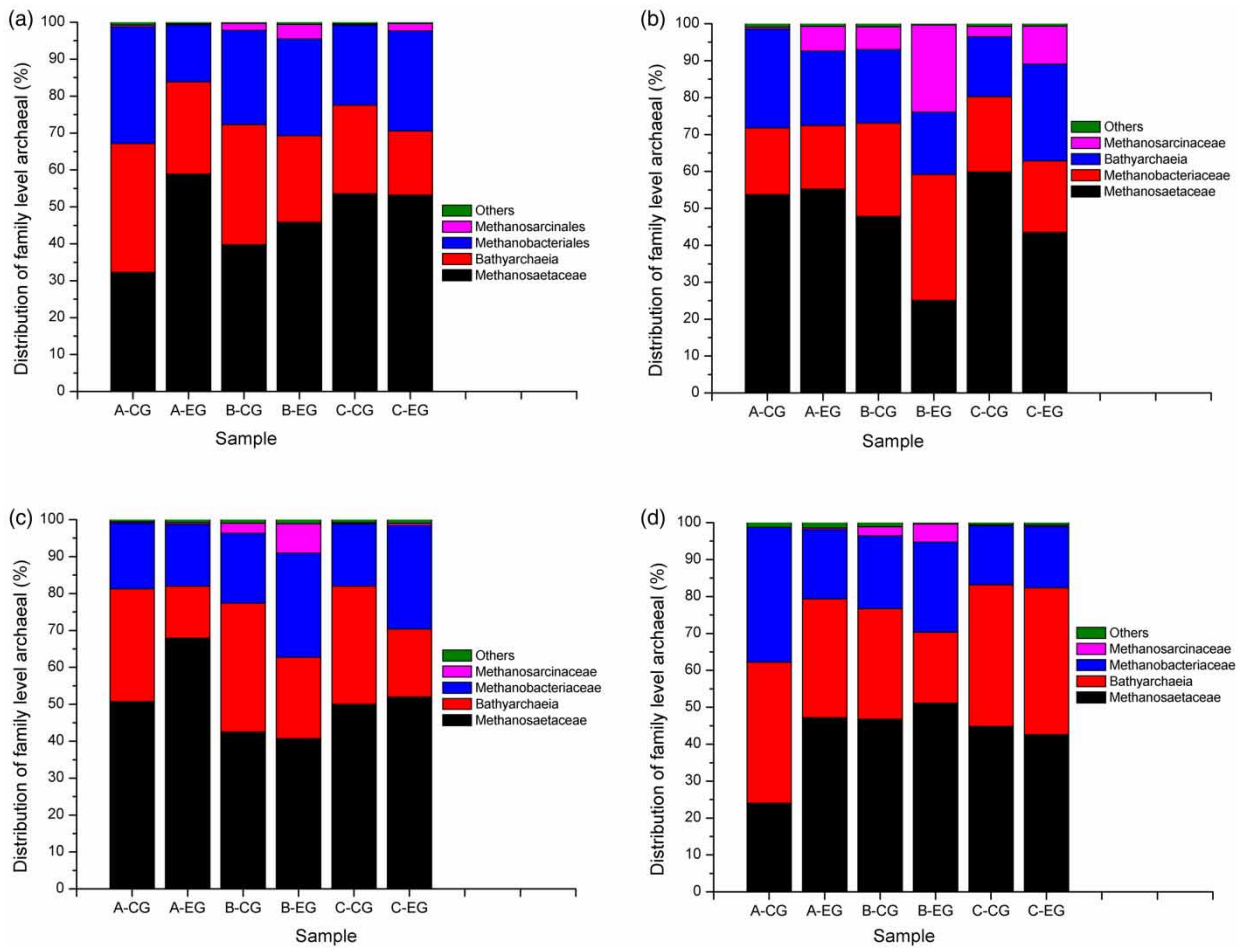


Figure 3 | Distribution of the main archaeal community abundance at the family level. (a) Low pH influent shock, (b) high OLR shock, (c) high ammonium shock, and (d) high sulfate shock.

subjected to low pH influent shock, the abundance of *Methanosarcinaceae* in EG B and C was higher than in the respective CGs, while that of *Bathyarchaeia* in EG A, B and C was 9.94, 9.06 and 6.68% lower than in the respective CGs. When the AD systems of the activated sludge were subjected to high OLR shock, the abundance of *Methanosarcinaceae* in EG A, B and C was 6.15, 17.39 and 7.46% higher than in each corresponding CG, the abundance of *Methanobacteriaceae* in EG B was 8.84% higher than in CG B, the abundance of *Methanosaetaceae* in EG B and C was 22.76 and 16.39% lower than in CG B and C. When the AD systems of activated sludge were subjected to high ammonium shock, the abundance of *Methanosarcinaceae* in each EG was higher than in the respective CGs, while the abundance of *Methanobacteriaceae* in EG B and C was 9.26 and 11.16% higher than in the respective CGs, the abundance of *Methanosaetaceae* in EG A was 17.22 higher than in CG A, and the abundance of *Bathyarchaeia* in each EG was lower than in their respective CGs. When the AD systems of the activated sludge were subjected to high sulfate shock, the abundance of *Methanosarcinaceae* in each EG was higher than in their respective CGs, while the abundance of *Bathyarchaeia* in EG A and B was 5.96 and 10.71% lower than in their respective CGs.

Results of archaeal community PCA in activated sludge of AD systems are shown in Figure 4. The archaeal community of activated sludge was affected by stress shock to some extent. When the AD systems of activated sludge were subjected to high OLR shock, the archaeal community of sludge B-EG was different from sludge A-EG and C-EG, that of sludge A-EG and C-EG was similar. When the AD systems of activated sludge were subjected to high ammonium and sulfate shock, there was little difference in archaeal community of sludge A and C, while there was great difference in archaeal community of sludge B.

The archaea community in all of the anaerobic activated sludge samples was dominated by *Methanosaetaceae*, *Methanobacteriaceae*, *Methanosarcinaceae* and *Bathyarchaeia*. *Methanosaetaceae* is strict aceticlastic methanogens that are adapted to low acetate concentrations. *Methanobacteriaceae* is hydrogen-utilizing methanogens. *Methanosarcinaceae* is metabolically flexible and have a high level of metabolic capability, including acetoclastic and hydrogenotrophic methanogenesis. *Bathyarchaeia*, which is *Crenarchaeota,* was reportedly closely correlated with environmental properties such as pH, Na^+ and Cl^- and play a role in regulation of ecological functions in stressed habitats (Wang *et al.* 2019b). When the AD system was subjected to stress shock, the relative abundance of *Methanosarcinaceae* in the activated sludge was higher in almost all of the EGs than in the CGs. These findings suggested that *Methanosarcinaceae* was better able to cope with stress shock, which may have been because of the cluster structure and its flexible metabolic pathways. These findings agreed with those previous studies that showed *Methanosarcinaceae* had better resistance to high ammonium and VFA (Regueiro *et al.* 2016). The relative abundance of *Methanobacteriaceae* in the activated sludge of EG B was higher than in CG B under stress shock. The relative abundance of *Methanosaetaceae* in the activated sludge of EG B was lower than in CG B under high OLR and high ammonium shock, which had a greater impact on the AD system. These findings indicated that the metabolism of hydrogenotrophic methanogenesis was enhanced in the AD systems of EG B under severe stress shock,

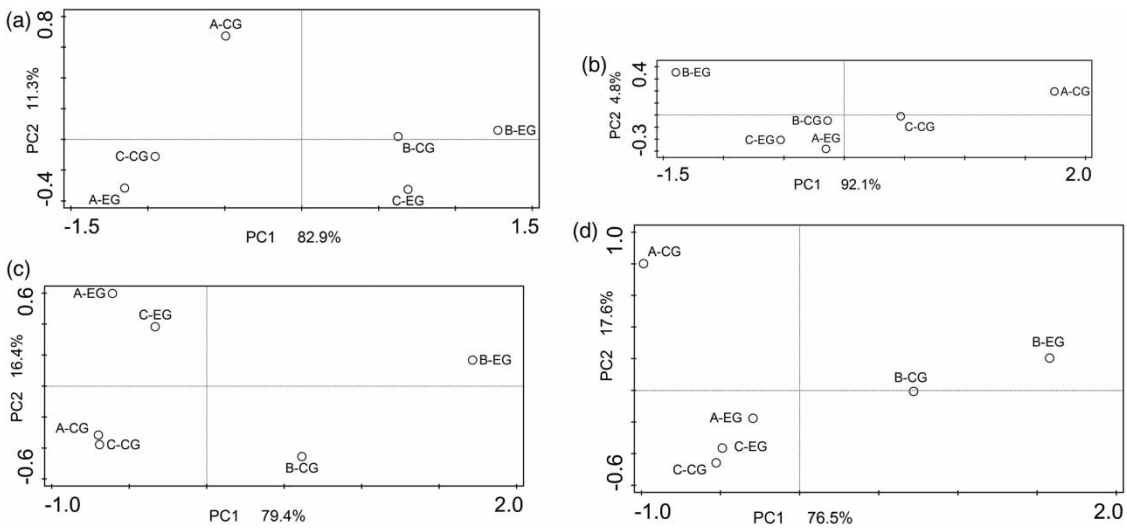


Figure 4 | Principal component analysis based on archaeal community. (a) Low pH influent shock, (b) high OLR shock, (c) high ammonium shock, and (d) high sulfate shock.

which was consistent with the results of previous studies. Previous studies have reported that the metabolism of acetoclastic methanogens was inhibited and the subsequent oxidation of acetate to H_2 and CO_2 provided hydrogen for the reduction of CO_2 by hydrogenotrophic mechanisms when the anaerobic system underwent stress shock and the metabolic pathway shifted from acetoclastic methanogenesis to hydrogenotrophic methanogenesis (Zhao *et al.* 2013; Buhlmann *et al.* 2019).

As shown in section 3, there was no significant effect on the performance of EGs after 25 days of low pH influent shock, indicating that the acclimated anaerobic activated sludge had good buffering capacity and was resistant to low strength pH shock. In the long acclimation process, activated sludge adapted to the changes of the external environment through its own metabolism regulation, and gradually formed stable and metabolically diverse functional microbial communities. The functional microbial community made the anaerobic system have a good buffer capacity. In addition, compared with the CGs, the relative abundance of the main functional bacteria in the activated sludge of EGs changed. Wang *et al.* (2019c) found that alkaline/acid treatment shifted the microbial communities of anaerobic activated sludge. Accordingly, further study is needed to determine if long-term low pH influent will have a significant impact on the performance of AD systems. Based on the performance of the AD systems under high OLR shock, the activated sludge B had the best resistance to high OLR, followed by C and then A. As shown in Figures 1(b) and 3(b), the variations in the relative abundance of some bacteria (*Rikenellaceae* and *Ruminococcaceae*) and methanogens (*Methanosaetaceae* and *Methanobacteriaceae*) in each activated sludge differed between the EGs and CGs. The variations in activated sludge B and C were similar, with the greatest difference occurring between B and A. These findings suggested that the microbial community in anaerobic activated sludge acclimated by different feeding patterns changed to different degrees when subjected to stress shock, and the variations in relative abundances of functional microorganisms under stress shock was the reason for the different stress resistance of the three activated sludges.

4. CONCLUSION

The acclimated anaerobic activated sludge has good resistance to low pH influent and high sulfate shock, but poor resistance to high OLR and high ammonium shock. The stable and metabolically diverse functional microbial community in the acclimated activated sludge made it respond well to low pH influent and high sulfate shock. The increased abundance of SRB associated with sulfate metabolism also made activated sludge well resistant to high sulfate shock. The high OLR and high ammonium beyond the capacity of microorganisms in activated sludge led to the decrease of microbial activity, and the increase of the concentration of ammonia nitrogen and VFA in the AD system, which further inhibited the growth and metabolism of the microbial community, so that the resistance of activated sludge to high OLR and high ammonium shock decreased greatly. Anaerobic activated sludge acclimated by semi-continuous feeding pattern has better resistance to high OLR shock than that acclimated by continuous feeding pattern and pulse feeding pattern. Under stress shock, the microbial community structure and abundance of specific functional microorganisms in the activated sludge acclimated by different feeding patterns varied. Under stress shock, the relative abundance of *Methanosarcinaceae* in the anaerobic activated sludge increased, and the variation in the relative abundance of specific functional microorganisms led to differences in the resistance of the anaerobic activated sludge.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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