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Influence of tetracycline contents on the performance of expanded granular sludge bed and its microbial community structure

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ABSTRACT

Antibiotics wastewater is normally treated by anaerobic biological technology, while the performance is always not satisfied due to the inhibitory effects of high level antibiotics. Here, we investigated the quick start-up process for tetracycline wastewater treatment in expanded granular sludge bed (EGSB) reactor and also the influence of tetracycline content on performance and microbial community structure. The results indicated that gradual increase of tetracycline content could keep the stable operation of EGSB system, while cause slight inhibition. With tetracycline content increasing to 210 mg/L, chemical oxygen demand (COD) removal decreased from 96.7% to 73.3%, tetracycline removal slightly decreased from 84.6% to 81.6%, while biogas production decreased from 6.1 to 4.7 L/d with methane production rate of 208 to 175 mL/g COD. Long-term acclimation could eliminate the inhibition by tetracycline and its metabolic products to recover the microbial activity. Bacillus brevis, Cocci and Clostridium became the main bacteria with increase of tetracycline contents to form a compact and multi-layer structure of sludge. Tetracycline addition resulted in the increase of Spirochaete and Synergistetes, which might promote the removal of tetracycline. Tetracycline addition decreased the total contents of methanogens and caused less methane production. It provided an effective start-up method for anaerobic treatment of antibiotics wastewater.

Keywords: Tetracycline contents; Expanded granular sludge bed; Pollutants removal; Methane production; Microbial community structure

1. Introduction

Antibiotics were one group of important pharmaceuticals and widely used to prevent and cure the infectious diseases in humans and animals [1]. It was also extensively used as growth promoter for animals such as poultry, cattle, pig and fish [2]. The production and consumption of antibiotics in China was largest in the world [3]. It was up to 248 and 162 kilotons in 2013, respectively, and the total emission of 36 target antibiotics into water and soil environments reached 53.8 kilotons [4]. Widespread utilization of antibiotics inevitably resulted in large amounts of antibiotic residues releasing into ecological environment as the parent compounds or their active metabolites [2]. The antibiotics entering into environment were mainly from hospital wastes, pharmaceutical industries, veterinary drugs and also municipal wastewater [5]. The concentration of antibiotics was detected ranging from ng/L to a few μg/L in WWTPs, and a higher level of mg/L in some point sources such as the effluent from pharmaceutical industry and hospital [6]. Therefore, antibiotics had been frequently found in multimedia environment including water, sediments, soil, sludge, etc, which promoted the development and transportation of antibiotic resistance genes (ARGs) and bacteria, causing serious ecological risks to environmental safety and human health [7,8].

Antibiotics wastewater was normally refractory with the characters of high chemical oxygen demand (COD), low biodegradability, terrible odor, high chroma, high biological

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toxicity and high sulfate contents because antibiotics production need use lots of reactants, solvents, catalysts etc [9]. Numerous methods, including advanced oxidation processes (AOPs), biological process, activated carbon adsorption, bio-electrochemical system, and membrane processes, etc had been used for antibiotics wastewater treatment [10–13]. Though AOPs such as ozonation, photocatalytic degradation, UV/H_2O_2, H_2O_2/O_3 and Fenton oxidation could effectively decompose antibiotics, they had the problems of high cost due to the waste of oxidants by reacting with the coexisting bulk pollutants in antibiotics wastewater [14,15]. Due to the low operating costs and high efficiency, biological treatments turned out to be a promising and attractive method for various antibiotics wastewater treatment, and were divided into aerobic and anaerobic types. For high concentration antibiotics wastewater, anaerobic process had the evident advantages over aerobic process in terms of high load rate, lower energy consumption, biogas production and less sludge production.

Various anaerobic treatment systems had been used to effectively treat antibiotics wastewater. Yi et al. [14] found that UASB reactor could effectively treat oxytetracycline production wastewater with COD removal efficiency of 69%–83% at the organic loading rate of 3.3–5.9 kg COD/(m·d) [14]. Anaerobic membrane bioreactors were reported as a promising technology for emerging antibiotics removal in wastewater with the removal efficiency of COD and antibiotics more than 90% [16]. Previous study reported that anaerobic digestion could effectively treat pharmaceutical wastewater containing tetracycline in semi-continuous lab-scale fermenters with removal rate of 14%–89% [17]. However, the presence of high antibiotics contents in wastewater could inhibit the COD removal and methane production in anaerobic biological treatment system due to the bacteriostatic effects of antibiotics [18]. Aydin et al. [19] also found that various tetracycline mixtures resulted in a remarkable synergistic effects on anaerobic digestion in anaerobic SBR and had negative impacts on homoacetogenic bacteria and methanogens by preventing bacterial protein synthesis. Lu et al. [20] found that low level of tetracycline (250 μg/L) could also negatively influence CH_4 and CO_2 production, and tetracycline presence could sensitively inhibit methanogenesis process. Therefore, anaerobic biological technology had drawbacks such as biological toxicity and slow start-up for high level of tetracycline wastewater treatment, and new methods were needed to solve these drawbacks.

The objectives of the present study were to (1) investigate the quick start-up method of expanded granular sludge bed (EGSB) for effective treatment of tetracycline wastewater; (2) clarify the influence of tetracycline content on operational efficiencies and biogas production; (3) resolve the impacts of tetracycline load on microbial community structure in EGSB. The results would provide us the theoretical basis and operational experience for the anaerobic treatment of antibiotics wastewater.

2. Materials and methods

2.1. Configuration of EGSB system

The lab-scale EGSB system used in this study consisted of a tetracycline wastewater tank, EGSB main body, temperature controlling equipment, wet gas flow meter and desulfidation system. The main body of EGSB made with strong plexiglass had an effective volume of 4 L with the internal diameter of 8.0 cm and height of 120 cm. The operational temperature in reactor was kept constant at 35°C ± 1°C with four hoop heating resistors (200 W) attached the surface of reactor. pH and oxidation–reduction potential (ORP) probes were equipped in reactor to real-timely measure the pH value and ORP. The up-flow velocity in EGSB was kept constant at 0.7 m/h by controlling the recirculation ratio of 20:1. A gas–liquid–solids separator was installed at the top of column and used to separate biogas and solids. The gas collection tube was connected at the top of EGSB and a glass bottle (1 L) containing desulfurizer was used to remove H_2S from biogas. Then, a wet gas meter was employed to detect the daily biogas production.

2.2. Inoculums and wastewater composition

Anaerobic granular sludge was used as the seed sludge, which was obtained from UASB reactor of beer industry in Harbin, China. The initial granular sludge showed black granules with the size of 0.5–5.0 mm in diameter and 0.02–0.04 m/s of the sedimentation velocity. The mixed liquor suspended solid (MLSS) of seed sludge was 22.38 g/L and mixed liquor volatile suspended solid (MLVSS) was 14.1 g/L. Sludge was stored at 4°C under anaerobic condition before use. The seed amount was 66.7% (v/v) of the effective volume at the start of experiments.

According to the main composition of real tetracycline wastewater, synthetic wastewater was used in laboratory. Saccharose, NH_4Cl and K_2HPO_4 was used as carbon, nitrogen and phosphorus source, respectively, while tetracycline and Na_2SO_4 were used to provide antibiotics and sulfate in wastewater. COD, tetracycline, sulfate and NH_4^+–N were adjusted to in range of 3,000–6,000, 0–250, 0–1,000 and 75–800 mg/L at the different operation stage, respectively, as shown in Table 1. Moreover, 10 mL trace element solution were added (per liter) to supply necessary nutrient for microbial growth. The composition of trace element solution (in g/L) was as described in previous study [21]. The pH of synthetic wastewater was adjusted to 8.0 ± 0.3 using Na_2CO_3 and HCl solution.

2.3. Experimental procedure

The main objective of this study was to rapid start-up and high efficient treatment of high-concentration tetracycline wastewater. The operation process included start-up, load increasing and stable stage. At start-up stage, influent COD was started at 3,000 mg/L with the organic loading rate (OLR) of 2.53 kg COD/m^3·d. At loading increasing stage, COD was increased to 5,000 mg/L with OLR of 4.33 kg COD/m^3·d, and tetracycline content was gradually increased from 0 to 210 mg/L. At stable stage, COD was kept at 6,000 mg/L with OLR of 5.06 kg COD/m^3·d and tetracycline of 250 mg/L. The hydraulic retention time (HRT) of EGSB was set as 24 h. The whole operation lasted for 183 d.

2.4. Analytical methods

The influent and effluent samples of EGSB were sampled every day to evaluate the performance of reactor.

Table 1
Water quality indexes and operation parameters of EGSB reactor

Stage		COD (mg/L)	TC (mg/L)	SO_4^{2-} (mg/L)	NH_4^+–N (mg/L)	HRT (h)	NV (kg·COD/m³·d)
Start-up stage	I	3,000	0	0	75	24	2.53
	II	5,000	0	0	125	24	4.33
Load increasing stage	III	5,000	53	210	260	24	4.33
	IV	5,000	105	418	396	24	4.33
	V	5,000	210	835	667	24	4.33
Stable stage	VI	6,000	250	1,000	800	24	5.06

Water quality parameters such as pH, ORP, COD, total organic carbon (TOC), SO_4^{2-}–S, S^{2-}, MLSS and MLVSS were determined according to the standard methods [22]. Volatile fatty acids (VFAs) of effluent samples were measured with a gas chromatograph (Agilent GC 7890A, USA) with flame ionization detector (FID) equipped with a HP-INNOWAX column [23]. The tetracycline content was determined with a liquid chromatograph (LC-2010, Shimadzu, Japan) with variable wavelength UV/VIS detector equipped with a C18 column (4.6 mm × 150 mm) [21]. The biogas production was measured with a wet gas meter (LML-1, China), and the composition (H_2, CH_4 and CO_2) were determined with a gas chromatograph (Agilent 6890A, USA) [24]. The morphology and structure of granule sludge at different stages were analyzed with scanning electron microscopy (SEM) images, and the detail procedure for SEM treatment was shown in previous study [25].

The bacterial and archaea communities in EGSB at start up (day 9) and stable stage (day 180) was investigated by illumina high-throughput sequencing. The extraction and amplification of sludge DNA were detailedly described in previous study [21]. After amplification, samples were conducted pyrosequencing on the Illumina MiSeq platform (Shanghai Majorbio Bio-Pharm Technology Co., Ltd., China). Operational taxonomic units (OTUs) with identities of 97% were identified with MOTHUR program. The alpha diversity indices including species richness, Shannon diversity index, abundance-based coverage estimator (ACE), Chao 1 and coverage were used to evaluate the biodiversity of microbial community.

3. Results and discussion

3.1. Performance of EGSB system under different tetracycline contents

The pH and ORP were key parameters affecting the growth and development of anaerobes, especially methanogens. Fig. 1 shows the ORP and pH changes of influent and effluent in EGSB at each stages. ORP in I-III stage were kept at about –473 (bottom) and –467 (upper) mV in reactor, while it increased to –437 (bottom) and –443 (upper) mV at IV stage, and finally decreased to –485 (bottom) and –476 (upper) mV. Due to the higher height-diameter ratio of EGSB, the upper ORP was much lower than that at the bottom. The influent pH was almost stabilized at range of 7.9–8.4, while the effluent pH was kept at range of 7.3–8.1, which was slightly lower than that in influent caused by the acidification of tetracycline wastewater. The optimum pH and ORP for methane producing archaea was reported at 6.5–8.0 and lower than –330 mV. It indicated that pH and ORP in present study created a more advantageous condition for anaerobic microorganisms.

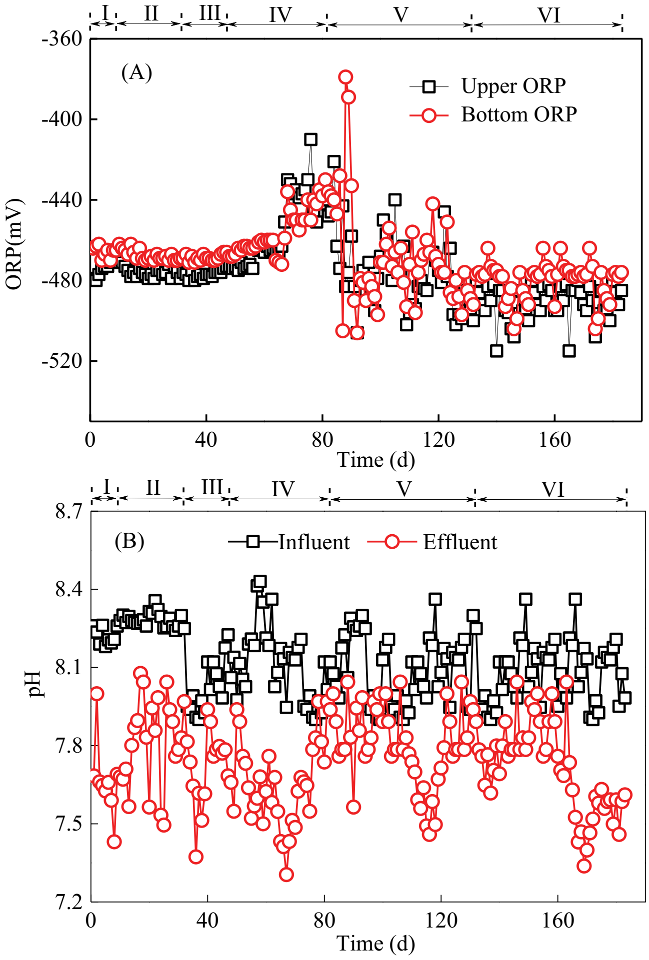

Fig. 1. Changes of ORP and pH with time during the start-up and stable stage of EGSB.

Fig. 2A shows the COD variations at different tetracycline contents. At start-up stage, the influent COD was stabilized at 3,053 ± 34 mg/L with a removal efficiency of 97.3%. After it increased to 5,074 ± 180 mg/L (II stage), the removal rate was almost kept constant at 96.7%, indicating that appropriate enhancement of organic loading did not influence performance of EGSB. However, tetracycline addition evidently inhibited COD removal. When tetracycline contents increased from 0 (II stage) to 210 mg/L (V stage) with total COD of 5,000 mg/L, COD removal decreased from 96.7% to 73.3%. At stable stage, COD removal further decreased to 70.3% by enhancing of tetracycline and COD to 250 and 6,000 mg/L. At each stage of III–VI, tetracycline addition could firstly decrease COD removal and then recover to a stable level. Tetracycline and the produced metabolic products might produce toxicity to the anaerobic microorganism, while long-term acclimation could eliminate the inhibition and recover the microbial activity. Fig. 2B shows influent TOC started at approximate 1,121 ± 19 mg/L (I stage), then increased to 1,885 ± 144 mg/L (II–V stage) and finally increased to 2,216 ± 89 mg/L (II–V stage). The corresponding removal efficiency of TOC decreased from 97.2% to 81.3% and finally 77.8%, which had the similar trend with COD.

Fig. 2C shows the changes of sulfate removal and sulfide production in reactor. Sulfate removal could rapidly increase to 93.8% after 14 d operation at III stage, and sulfide was produced at approximate 55.8 mg/L, indicating the sludge had higher sulfate reducing activity. When sulfate content increased from 70.6 mg/L to 140.1, 279 and 335 mg/L, the sulfate removal efficiency kept almost constant at 95.3%, 94.7% and 94.6%, respectively. It implied that enhancing sulfate load had little impacts on sulfate reduction process, though an abrupt decrease of sulfate removal occurred at initial of each stage. Compared with sulfate and VFAs profiles (Fig. 3), the increase of tetracycline and its metabolites did not cause evidently inhibition on sulfate reducing bacteria (SRB), while evidently influence the activity of acetogenic bacteria and methanogens, indicating SRB was predominant in anaerobic sludge. Sulfide contents increased from 55.8 to 105.1, 207.1 and 243.3 mg/L by increasing sulfate from 70.6 mg/L (III stage) to 335 mg/L (VI stage).

Fig. 2D shows the removal situation of tetracycline in EGSB. At III stage, the influent tetracycline concentration was kept constantly at 50 mg/L with the removal efficiency of approximate 84.6%. Further increasing of initial tetracycline content to 105, 210 and 210 mg/L caused the removal efficiency slightly decreasing to 82.3%, 81.7% and 81.6% at the stable part of each stage, indicating tetracycline content almost had no impacts on its removal. However, tetracycline removal evidently decreased to a valley bottom at the start of IV–VI stage, and then it recovered to the previous level. The decrease might be caused by the inhibition effects of anaerobic metabolic products of tetracycline, while long-term acclimation could reduce the inhibition, which corresponded

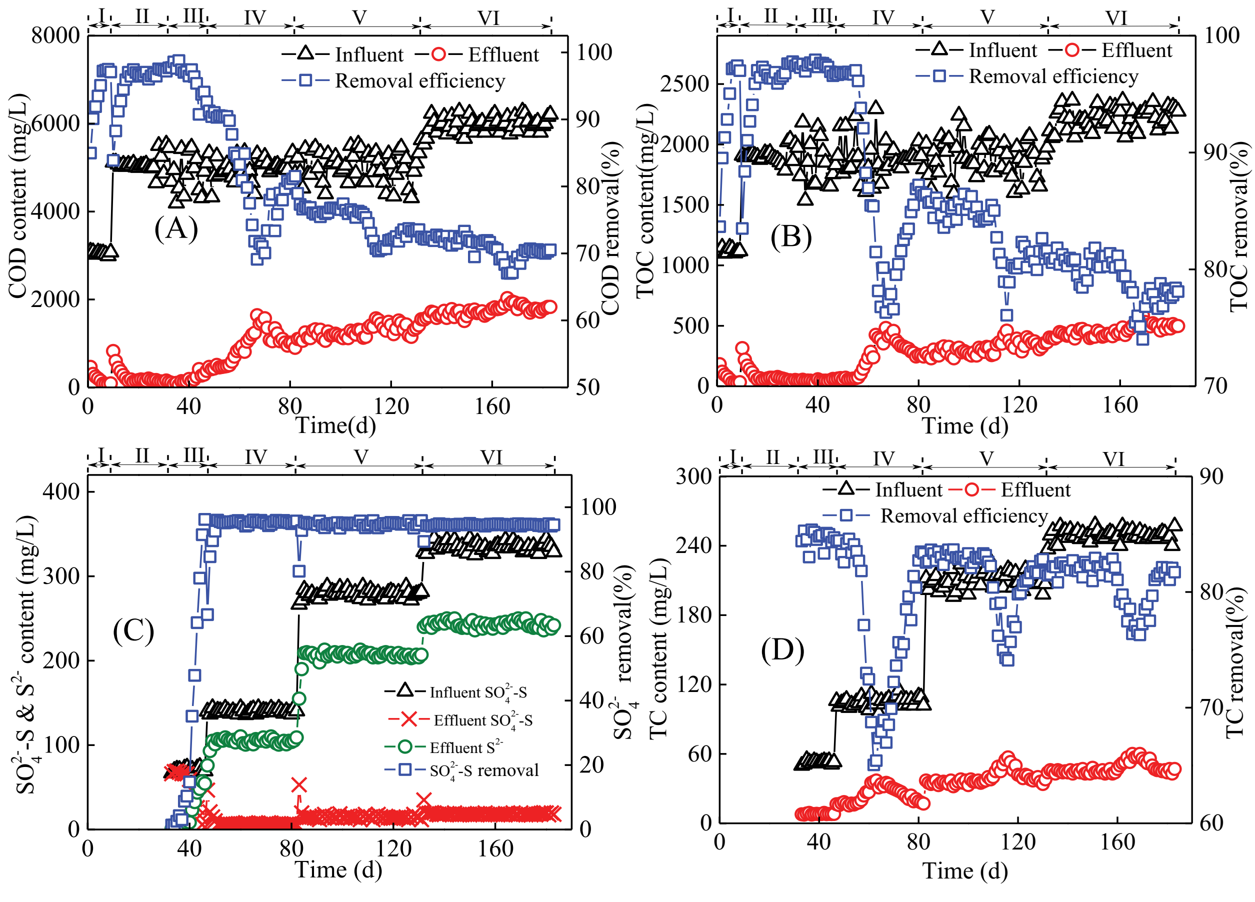

Fig. 2. Changes and removal of various water qualities during the start-up and stable stage of EGSB. (A) COD removal, (B) TOC removal, (C) sulfate removal and sulfide production and (D) TC removal.

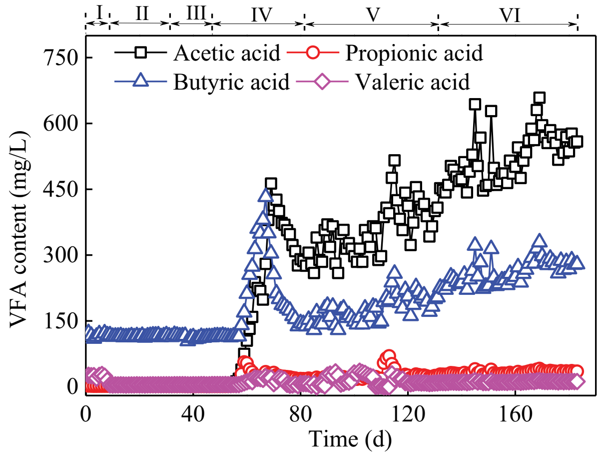

Fig. 3. Changes of VFAs during the start-up and stable stage of EGSB.

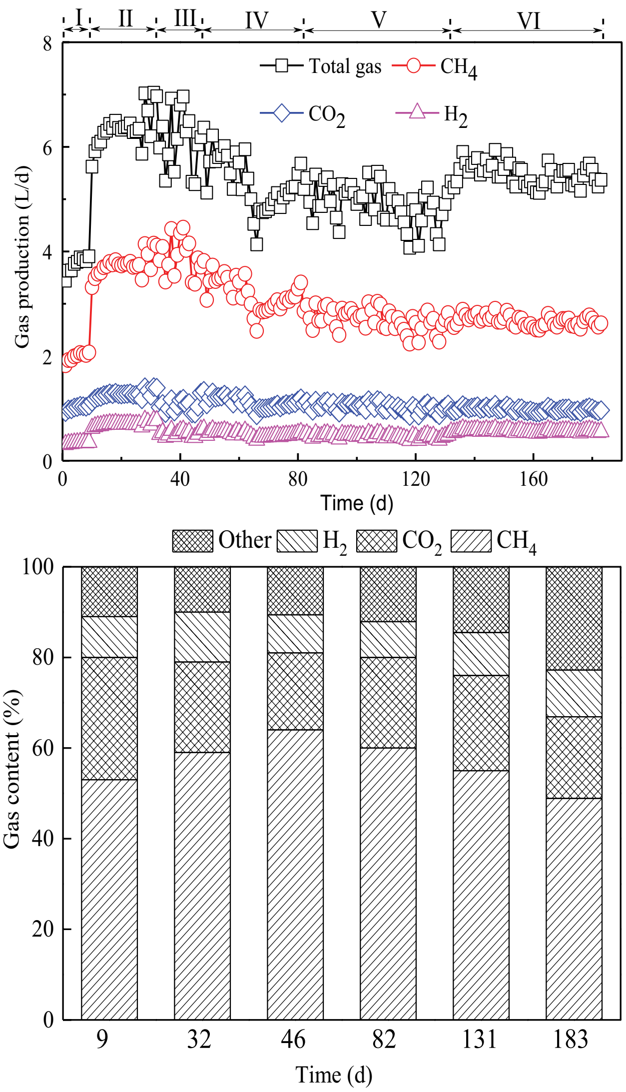

Fig. 4. Changes of daily gas production and gas contents at each stage of EGSB.

to COD removal. The influence content also decreased with tetracycline concentration. For example, the valley removal was 65%, 74% and 76% at the tetracycline content of 105, 210 and 250 mg/L, respectively. Due to tetracycline removal mainly relied on hydrolysis fermentation bacteria, above results reflected that hydrolysis fermentation process was not influenced by higher levels of tetracycline.

The changes of VFAs including acetic acid, propionic acid, butyric acid and valeric acid during the whole operation process are shown in Fig. 3. Acetate acid and butyric acid were the main VFAs in aqueous solution, which was changed evidently with tetracycline contents. At I-III stage, acetate acid and butyric acid were kept at 4.9 ± 0.5 and 115 ± 3 mg/L, indicating that EGSB operation was not influenced by increasing COD and tetracycline to 5,000 and 53 mg/L, respectively. However, when tetracycline content increased to 105 mg/L by keeping COD constant, they rapidly enhanced to 463 and 433 mg/L at 69 d, then decreased to 282 and 141 mg/L at the end of IV stage, indicating that acclimatization could reduce the inhibitory effects. Further enhancing tetracycline to 250 mg/L resulted in the accumulation of acetate acid and butyric acid increasing to approximate 553 and 279 mg/L. Above results implied that higher tetracycline addition and its metabolites could inhibit the activity of methanogens using acetic as substrate and bacteria using butyric as substrate, resulting in their accumulation in system. Tetracycline addition did not change the metabolic type but a slow consumption of VFAs, which increased with tetracycline contents and corresponded to the methane production status. Butyric acid and valeric acid were kept at low level during the whole operation, while they increased from approximate 1.7 and 7.8 mg/L to 28.7 and 12.3 mg/L after adding tetracycline above 53 mg/L. By comparison, tetracycline inhibited microorganism using acetic and butyric as substrate more serious than that using other two acids.

3.2. Biogas production and composition

As shown in Fig. 4, the composition and contents of biogas were measured to assess the anaerobic digestion process. Biogas production rate stabilized at 3.7 ± 0.2 L/d with methane content of 53% at start-up stage, while it increased to 6.5 ± 0.3 L/d with methane content of 59% by enhancing COD to 5,000 mg/L. The corresponding methane production rate increased from approximate 170 to 193 mL/g COD, indicating that more substrate could promote biogas and methane production. However, biogas production decreased to 6.1 ± 0.6, 5.2 ± 0.2 and 4.7 ± 0.4 L/d by adding tetracycline content of 50–210 mg/L, while the corresponding methane production rate slightly increased to 208 mL/g COD (III stage) and then decreased to 175 mL/g COD (V stage), implying that tetracycline addition could inhibit the activity of anaerobic bacteria and methanogen. At VI stage, biogas production further raised to 5.4 ± 0.2 L/d by enhancing COD and tetracycline content to 6,000 and 250 mg/L, while methane production was almost kept constant at 2.7 ± 0.1 L/d with the production rate decreasing to 158 mL/g COD, indicating that further increasing of COD could not enhance methane production at higher tetracycline level. Moreover, CO_2 and H_2 almost kept constant at 1.1 ± 0.1 and 0.5 ± 0.1 L/d with the gas content in range of 18–27% and 8.4%–11% during the whole operation, indicating that enhancing COD and tetracycline concentration did not evidently influence their production. The other composition of biogas increased from 10% to 22.8% with the raising of tetracycline content, and it might be NH_3 producing from ammonium, which was enhanced evidently in the wastewater.

3.3. Sludge characteristics

An obvious difference could be seen from SEM pictures of anaerobic sludge in EGSB at different tetracycline contents (Fig. 5). Bacillus longus were dominated in the inner and surface of sludge, forming a loose and rough surface of sludge with channel-like structure before tetracycline

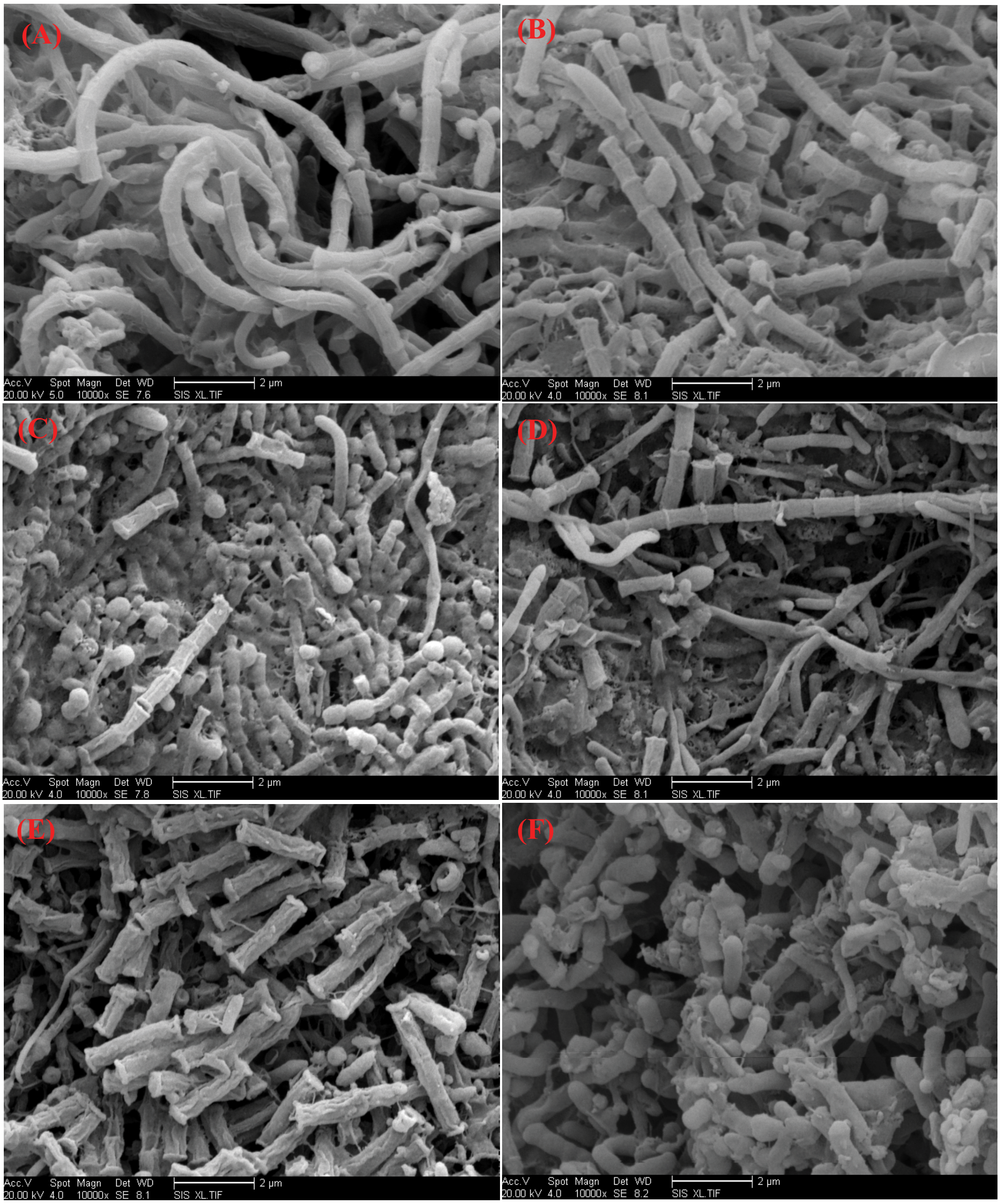

Fig. 5. SEM pictures of anaerobic sludge in EGSB system at different operation stage. (A) Start-up stage (I), (B–E) load increasing stage (II–V) and (F) stable stage (VI). The size bar corresponded to 2 μm.

addition. However, Bacillus brevis, Cocci and Clostridium became the main bacteria with increase of tetracycline contents, forming a compact and rigid appearance with a multilayer structure in the granular sludge. Higher tetracycline could cause toxicity to microorganism in sludge, and further induce more EPS-like substances production on the surface of sludge to resist the cytotoxicity of tetracycline and its metabolite. EPS could clog the channel-like structure to form the more smooth surface and densely packed aggregates, and also play a crucial role in enhancing the mass and electron transfer between acidogens and methanothrix [21]. It could also promote the stability and floating of granular sludge in EGSB, which was also reflected from the pollutant removal and gas production [26].

3.4. Microbiological community structure

The microbial community of anaerobic sludge at start-up and stable stage were determined by Illumina high-throughput sequencing. The alpha diversity indexes are shown in Table 2. The Chao 1 and ACE index of bacteria increased from 637 and 706 to 749 and 932 with tetracycline contents increasing to 250 mg/L, indicating that tetracycline addition could increase the number of species [27]. The Shannon index also increased from 3.64 to 4.27, indicating the diversity and complexity of community increased after tetracycline addition. The coverage values of bacteria and archaea were all above 0.99, indicating that the used sampling tool was sufficient to reflect the community composition of EGSB system.

The bacterial community structure in the reactor at phylum, class and genus level are shown in Fig. 6A–C. At the phylum level (Fig. 6A), Chloroflexi (30.97%), Thermotogae (20.7%), Cloacimonetes (11.1%), Firmicutes (7.41%), Bacteroidetes (6.97%), Proteobacteria (6.33%) and Verrucomicrobia (2.94%) were the dominant bacteria at start-up stage, while Firmicutes (26.23%), Bacteroidetes (20.59%), Proteobacteria (18.69%), Spirochaete (11.4%) and Chloroflexi (6.39%) predominated at the stable stage. Bacteria in Firmicutes always contained endospore to resist the bad environment, and was reported as the dominant bacteria for COD removal, while some species of Bacteroidetes had the potential for DIET [28,29]. Several studies reported that Spirochaete and Synergistetes in AD system mainly related to the hydrolysis of complex organic substances and production of acetic acid [30,31]. tetracycline addition led to the relative abundance of Spirochaete (from 0.5% to 11.4%) and Synergistetes (from 1.46% to 3.19%) enhancing, and then might promote the removal of tetracycline. Due to hydrolysis, acidification and methanation was an integrated process in anaerobic conditions, the bacteria community changes of hydrolytic acidification would influence methanogenic process, which further affected the treating efficiency of wastewater. At class level (Fig. 6B), total of 18 bacterial classes were detected in sludge with 9 classes as the majority, including Anaerolineae, Thermotogae, Clostridia, Deltaproteobacteria, Bacteroidia, Subdivision3, Synergistia, Gammaproteobacteria and Spirochaetia. Tetracycline addition largely decreased the content of Anaerolineae and Thermotogae, while obviously increased of Bacteroidia, Clostridia, Spirochaetia, Deltaproteobacteria Gammaproteobacteria and Negativicutes. It was worth noting that tetracycline reduced the unclassified bacteria from 24.4% to 4.82% at class levels.

Fig. 6C shows a total of 18 genera were classified in sludge. Tetracycline addition changed the dominated genera from *Kosmotoga*, *Candidatus Cloacamonas*, *Longilinea*, *Levilinea* and *Bellilinea* at start-up stage to *Treponema*, *Desulfovibrio*, *Klebsiella*, *Longilinea*, *Syntrophomonas* and *Anaeroarcus* at stable stage. The abundance of *Treponema* increased from 0.1% to 8.9%, which was a chemoheterotrophic bacterium with the ability for complex compounds degradation [21]. It indicated that tetracycline addition improved the resistance ability of bacteria to tetracycline and its metabolite in system. *Levilinea* existed in normal anaerobic digestion systems and played a key role in hydrolytic fermentation and resistance to the toxicity of industrial wastewater [32]. It evidently decreased from 6.51% to 1.38%, and corresponded to the slight decrease of COD and tetracycline removal. *Klebsiella* had the resistance for antibiotics and could maintain the stable operation of EGSB at high tetracycline contents, which increased from 0.9% to 6.7%. *Desulfovibrio*, a sulfate reducing bacteria, could produce acetic acid and CO_2 by oxidizing the organic matters and utilize H^+ as electron acceptor for sulfate reduction [21]. It enhanced from 0.1% to 8.3%, indicating that tetracycline and sulfate addition promoted sulfate reduction process, which also was proven on the sulfate removal and sulfide production. *Syntrophomonas* and *Candidatus Cloacamonas* could convert various organic acids to hydrogen and acetate for subsequent methane production and performed syntrophic functions, which totally decreased from 13.7% to 5.1% and might inhibit methanogenic process [33,34].

Tetracycline addition could increase the Shannon diversity index of archaea from 1.48 to 1.66, while decreased the Chao1 richness index from 88 to 45, indicating tetracycline decreased archaea community. The archaea community structure in the EGSB at phylum, class and genus levels are

Table 2
The richness and diversity estimators of the microbiological sequences

	Sample name	Alpha-diversity						
		Effective sequences	OUT	Chao1	ACE	Shannon	Simpson	Coverage
Bacteria	$S_{start-up}$	23,332	438	637	706	3.64	0.0748	0.9937
	S_{stable}	36,487	431	749	932	4.27	0.0259	0.9958
Archaea	$S_{start-up}$	28,698	57	88	88	1.48	0.4157	0.9992
	S_{stable}	26,324	31	45	71	1.66	0.2494	0.9997

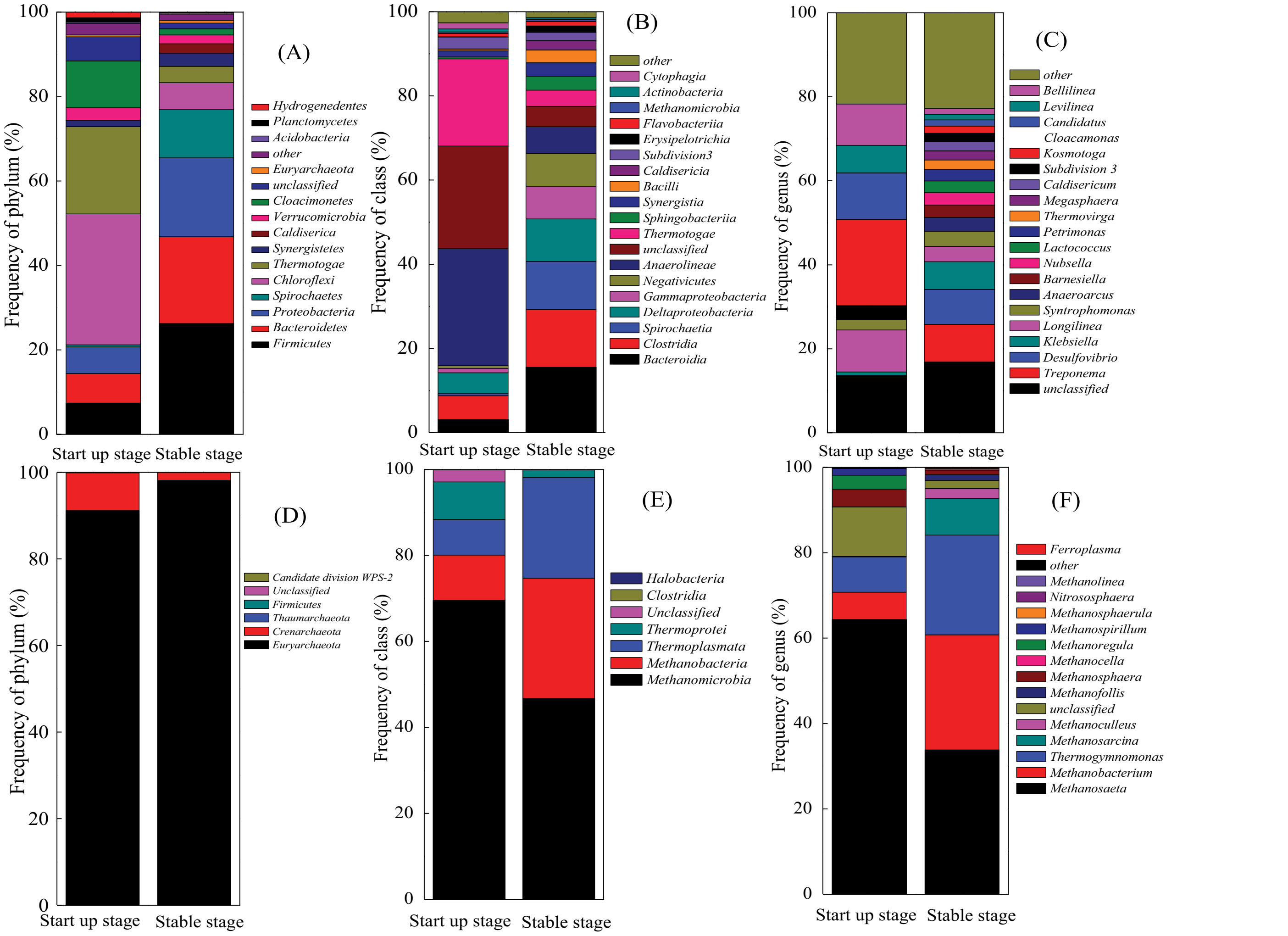

Fig. 6. Bacterial and archaea community structures of sludge in EGSB at phylum level (A, D), class level (B, E) and genus level (C, F) at start up stage and stable stage. The abundance is based on a percentage of the total effective sequences in the sludge samples.

shown in Fig. 6D–F. At phylum level (Fig. 6D), Euryarchaeota and Crenarchaeota were shown to be the two dominant archaea. Tetracycline addition increased the relative abundances of Euryarchaeota from 91.15% to 98.16%, while decreased Crenarchaeota from 8.74% to 1.82%. At class level (Fig. 6E), total of 6 archaeal communities were detected and the dominant sequences belonged to 4 classes. Tetracycline addition decreased Methanomicrobia and Thermoprotei from 69.57% and 8.74% to 46.73% and 1.82%, while increased Methanobacteria and Thermoplasmata from 10.55% and 8.27% to 27.97% and 23.43%, respectively. It was worth noting that the unclassified archaea decreased from 2.81% to 0.05%, and the relative abundance of the other archaeal classes was lower than 0.1%. The genus distribution of archaea community is presented in Fig. 6F. A total of 14 genera were presented in the two sludge samples. *Methanosaeta*, *Thermogymnomonas*, *Methanobacterium*, *Methanosphaera*, *Methanoregula* and *Methanospirillum* dominated in the sludge at start-up stage. Tetracycline addition could decrease the relative abundance of *Methanosaeta*, *Methanosphaera*, *Methanoregula* and *Methanospirillum* from 64.4%, 4.16%, 3.24% and 1.59% to 33.81%, 1.00%, 0.14% and 0.13%, while increase that of *Methanobacterium*, *Thermogymnomonas*, *Methanosarcina*, *Methanoculleus* and *Methanofollis* from 6.39%, 8.27%, 0.08%, 0.01% and 0.01% to 26.98%, 23.38%, 8.49%, 2.4% and 1.34%, respectively. It indicated that the dominant community was not affected by tetracycline addition. *Methanosaeta* were strict anaerobic filamentous organisms and could only use acetate as substrate for methane production. Meanwhile, *Methanosaeta* could provide a network to associate the other bacteria within the granule, and improve granulation of sludge to keep more stable performance of reactor [35]. Its reduction also corresponded to the accumulation of acetate and less methane production after tetracycline addition. *Methanobacterium* was hydrogenotrophic methanogens which could produce methane by reducing CO_2 with H_2 and formic acid [36]. This phenomenon was also consistent with the lower CO_2 and H_2 content at stable stage. *Methanosarcina* was the most versatile methanogen that could use different substrates, such as acetate, hydrogen and methyl containing groups to methane [37]. Moreover, the total contents of methanogens after tetracycline addition were decreased from 80.14% to 74.71%, leading to less methane production, which was also consistent with the methane data in reactor.

4. Conclusions

Anaerobic EGSB was a suitable system for tetracycline wastewater treatment, while its rapid start-up was crucial to the effective and stable operation. Gradual enhancing tetracycline content could cause slight inhibition for pollutant removal. 210 mg/L tetracycline could decrease COD and tetracycline removal by 23.4% and 3.0%, and also reduce biogas and methane production by 23% and 15.9%. The results indicated that stepwise enhancement of tetracycline content could eliminate the inhibition and recover the microbial activity. Tetracycline content could make the sludge form a compact and multi-layer structure, and change the microbial community composition. Increase of Spirochaete and Synergistetes could promote tetracycline removal, while decrease of total contents of methanogens was the main reason of less methane production.

Acknowledgements

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