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Effect of Diclofenac on the Production of Volatile Fatty Acids from Anaerobic Fermentation of Waste Activated Sludge

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Abstracts

In this study, the impact of diclofenac (DCF), an antiinflammatory drug being extensively used in human health care and veterinary treatment, on the production of volatile fatty acids (VFAs) from anaerobic fermentation of waste activated sludge (WAS) was investigated for the first time. Experimental results showed that when DCF concentration increased from 2.5 to 25 mg/kg total suspended solid (TSS), the maximum production of VFAs increased from 599 to 1113 mg COD/L, but further increase of DCF to 47.5 mg/kg TSS decreased VFAs yield to 896 mg COD/L. The mechanism investigation revealed that DCF had no effect on the hydrolysis process, promoted the process of acidogenesis, acetogenesis, and homoacetogenesis, but severely inhibited methanogenesis, leading to the accumulation of VFAs. Microbial community analysis showed that the addition of DCF could promote the relative abundance of VFAs (especially acetic acid) producers, which was well consistent with the results obtained above.

Key words: Sludge treatment; Anaerobic fermentation; Volatile fatty acids; Acidogenesis

1. Introduction

Activated sludge process is a commonly used treatment process in wastewater treatment plants (WWTPs). Although this process is very useful in protecting natural water bodies from pollution, large amounts of waste activated sludge (WAS) are inevitably produced as the major byproduct. WAS contains high levels of pathogens, heavy metals and other toxic and hazardous substances (Chen et al., 2018; Wang et al., 2018), and therefore requires to be effectively treated before its final disposal. On the other hand, WAS contains a lot of organics such as protein and carbohydrates, which make it a valuable resource (Wang et al., 2017a, 2017b).

Anaerobic fermentation has been recently considered a promising technology for WAS treatment, because this technology not only reduces the amount of WAS and kills pathogenic microorganisms but also produce various valuable products, such as volatile fatty acids (VFAs), which can serve as the preferred substrates for biological nutrient microbes and raw materials for microbial production of biodegradable plastics (Li et al., 2016; Wang et al., 2015a, 2017c; Zhao et al., 2015a; Zhao et al., 2016). In the past years, numerous efforts were paid on this topic (Carrere et al., 2010; Wang et al., 2013; Zhao et al., 2015b). Most of the studies in terms of VFAs production focused on the pretreatment method of WAS or the optimization of operation parameter of fermenter to enhance VFAs yield. For instance, Chen et al. investigated the effect of pH on sludge fermentation and found that alkaline pH favored the accumulation of VFAs because alkaline pH promoted sludge cracking and inhibited the activity of methanogens (Chen et al., 2007).

As the main byproduct of wastewater treatment, WAS not only contains high levels of biodegradable organics (e.g., proteins, carbohydrates, and polyhydroxyalkanoate) but also concentrates a variety of pollutants such as heavy metals and pharmaceuticals and personal care products (Xu et al., 2017; Wang et al., 2017d; Yi et al., 2017). These pollutants are usually absorbed onto the surfaces of sludge cells and finally enter into the WAS treatment system. It is known that WAS anaerobic fermentation is mainly dominated by

several biological processes (hydrolysis, acidogenesis, acetogenesis, and methanogenesis), with a variety of anaerobes such as acetogens, hydrogenogens, and methanogens involved. These anaerobes are likely to be affected by these entered pollutants, thus understanding and mitigating their potential toxicities to the WAS anaerobic fermentation have recently attracted increasing attention (Huang et al., 2017; Luo et al., 2016; Xu et al., 2017).

Diclofenac (DCF), an anti-inflammatory analgesic, is widely used in human health care and veterinary industry. It was reported that the annual consumption of DCF around the world was up to 940 t (Zhang et al., 2008), and this consumption would continue to increase in the future due to the increase of population and the rapid development of economy. The concentration of DCF in the environment is now generally at μg/L level. It was monitored that DCF concentration was 4.4 μg/L in the river and 8.5 μg/L in the drainage in Karachi, Pakistan (Scheurell et al., 2009). Ternes et al. demonstrated that DCF in wastewater was mainly absorbed in the solid phase in a municipal WWTP of German, and the concentrations of DCF in primary sludge and secondary sludge were respectively 7.02 mg/kg and 0.31 mg/kg (Ternes et al., 2004). Radjenovic et al. measured DCF at a concentration of 0.46 mg/kg in activated sludge and 0.2 mg/kg in primary sludge in a WWTP of Spain (Radjenovic et al., 2009).

DCF has a strong biological toxicity. Jiang et.al pointed out that 5 μg/L DCF damaged the microbial cell walls, which decreased total nitrogen removal in a sequencing batch reactor (Jiang et al., 2017). Using acetate as the model substrate, Symsaris et al. found that the IC_{50} value of DCF was 481-546 mg/L for methanogens (Symsaris et al., 2015). Despite these significant advances, these previous studies only focused on the toxicity of DCF to wastewater treatment, or anaerobic digestion of model substrate (e.g., acetate). To date, no information is available on the potential impact of DCF on anaerobic fermentation of real WAS for VFAs accumulation.

Compared with the wastewater treatment or anaerobic digestion of model substrate, anaerobic fermentation of real WAS contains different reactions (such as sludge disintegration, hydrolysis, and acidogenesis) and microbial community. These differences caused several questions required to be clarified: Does DCF at environmentally relevant level significantly affect anaerobic fermentation of WAS? If it does, how does DCF affect each process involved in anaerobic fermentation? How does DCF affect the diversity of microbial community and the abundances of key microbes in WAS anaerobic fermentation?

By addressing these questions, the aim of this work is to assess the potential impact of DCF on WAS anaerobic fermentation and to understand the underlying mechanisms of how DCF affects anaerobic fermentation of WAS. Firstly, the productions of VFAs from WAS fermentation with different DCF levels (i.e., 2.5, 7, 11.5, 25 and 47.5 mg/kg TSS) were compared. Then, details of how DCF affects VFAs accumulation were explored via assessing the impact of DCF on each process involved in sludge fermentation. Finally, microbial analyses were also performed to further understand the DCF's impact on VFAs production via analyzing the diversity of microbial community, abundances of functional microbes, and activities of key enzymes in different fermenters with different DCF dosages. The findings obtained in this study provides new insights into WAS anaerobic fermentation systems, fill the gap between the emerging pollutant, DCF, and anaerobic treatment of WAS, and might guide engineers to manipulate WAS treatment in the future.

2. Materials and Methods

2.1 Sludge and DCF

The WAS used in this study was taken from a secondary sedimentation tank of a municipal WWTP in Changsha, China. The collected WAS was first filtrated by stainless steel mesh (2.0mm) and concentrated at 4℃ for 24 h before being used for the following anaerobic fermentation tests. The main characteristics of the concentrated WAS were as follows: pH 6.8 ± 0.1, total suspended solids (TSS) 17550 ± 339 mg/L, volatile

suspended solids (VSS) 10821 ± 139 mg/L, total chemical oxygen demand (TCOD) 15253 ± 186 mg/L, soluble chemical oxygen demand (SCOD) 152 ± 11 mg/L, total protein 6577 ± 108 mg COD/L, total carbohydrate 851 ± 123 mg COD/L, and DCF 2.5 ± 0.3 mg/kg TSS. The extra addition of DCF in this study was purchased from Shanghai Chemical Pharmaceutical Co., Ltd., with a purity of 98%.

2.2 Effect of different DCF dosages on VFAs production from WAS anaerobic fermentation

In this batch experiment, five reproductive anaerobic reactors (working volume 1.0 L each) were established to assess the effect of DCF on VFAs production from sludge fermentation. According to the literature, the background value of DCF in the WAS is generally <7.02 mg/kg TSS (Ternes et al., 2004). In this test, two DCF concentrations (i.e., 2.5 and 7.0 mg/kg TSS) at environmentally relevant level were carried out. Since sludge DCF content might increase in the future due to its growing use, three higher concentrations of DCF, i.e., 11.5, 25.0 and 47.5 mg/kg TSS were also performed in this study. First, each reactor received 800 mL of the concentrated WAS, as mentioned above. Then 0, 0.1, 0.2, 0.5, 1.0 mL of DCF stock solution (0.6318 g/L) was respectively added to those reactors to control the concentration of DCF at 2.5, 7.0, 11.5, 25.0 and 47.5 mg/kg TSS at the beginning of the test. All the reactors were flushed with nitrogen gas for 5 min to remove the internal oxygen to ensure anaerobic environment, and then placed in an air-bath shaker (35 ℃) at stirring speed of 150 rpm. The whole anaerobic fermentation experiment was lasted for 12 d, and during which the impact of DCF on VFAs production was clearly obtained. It should be emphasized that no extra inoculum was added into the reactors, and WAS was therefore utilized for both fermentation substrate and inoculum in this study. The concentrations of SCOD, protein, carbohydrate, and VFAs in the liquid phase, DCF in both sludge and liquid phases, and hydrogen and methane in the gas phase were measured every day in the entire fermentation.

The total gas volume was determined by releasing the pressure in the bottle using a glass syringe (300

mL) to equilibrate with the room pressure according to the method reported before (Owen et al., 1979). The cumulative volume of hydrogen (or methane) gas was calculated by the following equation.

$$V_{H,i} = V_{H,i-1} + C_{H,i} \times V_{G,i} - C_{H,i-1} \times V_{G,i-1} \quad (1)$$

Where, $V_{H,i}$ and $V_{H,i-1}$ are respectively the cumulative volumes of hydrogen (methane) gas in the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are respectively the total gas volumes in the current and previous time intervals, and $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen (methane) gas detected by gas chromatography in the current and previous time intervals, respectively.

2.3 Long-term operation of the semi-continuous reactors for microbial analyses

Two semi-continuous reactors, with working volume of 1 L each, were performed in long-term operation to assess the impact of DCF on microbial community and key enzyme activities. The reactors were fed with the concentrated WAS containing either 2.5 or 25 mg/kg TSS DCF. All the operations were the same as depicted in the batch experiment above, except the semi-continuous operational condition depicted below. According to the results performed in Section 2.2, the maximum VFAs production was determined at 8 d fermentation in all the reactors. Therefore, the sludge retention time in both the semi-continuous reactors should be maintained at 8 d. On each day, 100 mL of fermentation mixture was manually taken out from the two reactors and then replaced with 100 mL of WAS containing either 2.5 or 25 mg/kg TSS DCF. After about 40 d operation, the yields of VFAs in both reactors were varied insignificantly, thus the microbial analyses were made.

2.4 Assessing the effect of DCF on each process in anaerobic fermentation

Anaerobic fermentation of WAS includes several processes such as disintegration, hydrolysis, acidogenesis, acetogenesis, homoacetogenesis, and methonogenesis processes. The rate of sludge disintegration can be evaluated by directly measuring the concentrations of soluble protein and carbohydrate

in the fermenters operated in Section 2.2. However, the amounts of intermediate or final products (e.g., VFAs, hydrogen, and methane) are relevant to all these processes. Thus, except for sludge disintegration, it is hard to clarify the impact of DCF on each process directly from the sludge fermentation systems. In the literature, the rates of these other processes are usually assessed by batch tests using model substrates (Duan et al., 2016; Mu et al., 2011). In this test, 25 replicate fermentation reactors with a working volume of 600 mL each were performed. These reactors were divided into five tests (namely, Protein-Dextran Test, L-glutamate-Glucose Test, Butyrate Test, H_2-CO_2 Test, and Acetate Test) with five in each.

Protein-Dextran Test: Each reactor received 500 mL synthetic wastewater and 100 mL the same inocula, which was withdrawn from the long-term reactor fed with 2.5 mg/kg TSS DCF sludge performed above. The synthetic wastewater contains 4.3 g/L bovine serum albumin (BSA, average molecular weight 67000, a model protein compound) and 0.8 g/L dextran (average molecular weight 23800, a model carbohydrate). The initial concentration of DCF in the five reactors was controlled at 2.5, 7, 11.5, 25, or 47.5 mg/kg TSS, which was consistent with that in the sludge fermentation systems in Section 2.2. pH in all the reactors was controlled at 7.0 ± 0.1. All other operations were the same as those depicted in Section 2.2. By measuring the degradations of the model compounds, the effect of DCF on hydrolysis process could be indicated.

L-glutamate-Glucose Test: The operation in this test was performed with the same method described in Protein-Dextran Test except that the fermentation substrates (i.e., BSA and dextran) in synthetic wastewater were replaced by L-glutamate and glucose.

Butyrate Test: The operational condition of this test was conducted the same as that described in Protein-Dextran Test except that the fermentation substrates in synthetic wastewater were replaced by 2.0 g/L sodium butyrate.

H_2-CO_2 Test: Each reactor received 100 mL same fermented sludge mixture as inocula and 500 mL tap

water, and the initial concentration of DCF was controlled to be the same as that in Protein-Dextran Test. Inocula used in this experiment were taken from the long-term reactor performed in Section 2.3. All the reactors were flushed with a combined gas (40% hydrogen, 10% carbon dioxide, and 50% nitrogen) for 5 min to ensure that they were filled with the synthetic hydrogen-containing gas. The pH in all the reactors was controlled at 7.0 ± 0.1. All the reactors were capped with rubber stoppers, sealed, and placed in an air-bath shaker (35 ℃) at stirring speed of 150 rpm.

Acetate Test: The operation of this test was carried out the same as depicted in Protein-Dextran Test except that 1.0 g/L sodium acetate was employed to replace BSA and dextran in synthetic wastewater.

2.5 DCF measurement

The variation of DCF during WAS anaerobic fermentation was detected via liquid chromatography, followed by tandem mass spectrometry (Agilent, 1260-6460, USA). Each day, 10 mL fermentation mixture was withdrawn from the fermentation reactor, then dried in a drying oven at 60-70℃. After weighing, the dry sludge was ground in a mortar and blended uniformly. An aliquot (0.05 g) of the sludge powder was dissolved by 8 mL methanol, then the sample slurry was ultrasonicated at 50℃ for 30 min. After centrifuging at 10000 rpm for 15 min, the supernatant was collected and then diluted to a final volume of 20 mL by ultrapure water. The diluted sample was introduced to an Oasis HLB cartridge (200 mg/3 mL, Waters, USA), which was preconditioned with 5 mL methanol and 5 mL ultrapure water, with a filtration rate of approximately 10 mL/min. After the cartridge was dried, the analyte was extracted by 5 mL acetonitrile. The extract was evaporated under 300 μL by flushing with weak nitrogen, and then 50 μL was injected into the liquid chromatography. Chromatographic separation was undertaken using a Symmetry C18 analytical column (50×2.1 mm, 3.5 mm particle size, Waters, USA). The mobile phase used acetonitrile as eluent A and ultrapure water as eluent B at a flow rate of 0.2 mL/min. The gradient elution started with 10 % eluent A and was held

for 2 min, then linearly increased to 95 % within following 3 min. After keeping these conditions at 95 % for 5 min, the initial phase condition was recovered within 6 s and was held for 10 min. A switching valve allowed sample to flow to the tandem mass spectrometry. The analyses were done in positive ion mode and the main parameters were as follows: drying gas temperature = 350 ℃, capillary voltage = 4.0 kV, drying gas flow rate = 11 L/min, and nebulizer pressure = 35 psi.

2.6 Analytical methods

Total COD, soluble COD, TSS, and VSS were analyzed according to standard methods (APHA, 1998). Protein and carbohydrate were determined by the Lowry-Folin method with BSA as the standard and the phenol-sulfuric method with glucose as the standard, respectively (Herbert et al., 1971; Lowry et al., 1951). The determination method of VFAs was consistent with the literature (Yuan et al., 2006), and the total VFAs was calculated as the sum of measured acetic, propionic, n-butyric, iso-butyric, n-valeric and iso-valeric acids. Hydrogen and methane fractions in the generated gas were determined using a gas chromatograph (GC112A, China), and the parameters were the same as the previous publications (Wang et al., 2015a, 2015b). The COD conversion coefficients are 1.42 g COD/g VSS, 1.07 g COD/g acetic, 1.51 g COD/g propionic, 1.82 g COD/g butyric, and 2.04 g COD/g valeric, 4 g COD/g CH_4, 1.5 g COD/g protein, 1.06 g COD/g carbohydrate, 8 g COD/g H_2 (Wang et al., 2015a). The measurement of key enzymes activities related to acetic acid production was according to the previous literature (Li et al., 2016). Differences in the diversity of microbial community referred to the production of VFAs between the two long-term operated reactors were conducted by Illumina HiSeq sequencing technique, and the detailed procedures were described in Supplementary data.

2.7 Statistical analysis

All experiments were conducted in triplicate in this study, and the results were expressed as mean ± standard deviation. An analysis of variance (p<0.05) was used to test the significance of results.

3 Results and discussions

3.1 Effect of DCF on VFAs production from WAS anaerobic fermentation

The effect of DCF on the production of VFAs from WAS anaerobic fermentation was shown in Fig.1a. It was found that the yield of VFAs showed a tendency of first rise and then decrease with the fermentation time. The maximum VFAs accumulation was 599 mg COD/L on 8 d, which was similar to the results obtained in the literature (Zhao et al., 2015c, 2016). When DCF appeared in the WAS fermentation system, the production of VFAs had undergone tremendous changes. The maximum yield of VFAs increased from 613 mg COD/L to 1113 mg COD/L when the content of DCF increased from 7 to 25 mg/kg TSS, further increase the concentration of DCF, however, had a negative effect on the accumulation of VFAs. For example, when the concentration of DCF increased from 25 to 47.5 mg/kg TSS, the maximum accumulation of VFAs decreased from 1113 to 896 mg COD/L. It should be noted that the addition of DCF did not change the optimum fermentation time, and the optimum fermentation time in all the fermenters was 8 d. Those experimental results clearly showed that the appropriate increase of the DCF concentration promoted the accumulation of VFAs, and the optimal amount of DCF was 25 mg/kg TSS, under which the maximum VFAs were 1113 mg COD/L and was 1.86 times of that in the 2.5 mg/kg TSS DCF reactor. The possible reason for the decrease in VFA concentration in the presence of excessive DCF may be attributable to the toxicity of DCF, which was previously reported that DCF was toxic to some microorganisms, such as *Pseudokirchneriella subcapitata* and *Vibrio fischeri*, by inhibiting their growth rates (Ferrari et al., 2003).

Fig.1b depicts the distribution of individual acid in each reactor at their optimum fermentation time. It is clear that the contents of propionic acid, butyric acid and valeric acid in each reactor were very similar. However, as for acetic acid, the presence of DCF significantly improved its concentration. The content of acetic acid increased from 274 to 623 mg COD/L with the increases of DCF from 2.5 to 25 mg/kg TSS.

Further calculations indicated that the increase in total VFA content (1113 - 599 = 514 mg COD/L) was mainly due to the increase in acetic acid content (623 -274 = 349 mg COD/L) when the concentration of DCF is increased from 2.5 to 25 mg/kg TSS. It is therefore necessary to explore how DCF induced an increase in acetic acid content in the following discussion.

3.2. Effect of DCF on methane production in the initial 8 d

The specific production rate of methane with different DCF levels in each day of the initial 8 d fermentation is shown in Fig.2. It can be seen that the production rate of methane varied a lot when DCF level increased from 2.5 to 47.5 mg/kg TSS. For example, it was respectively 10.1 ± 0.5, 9.8 ± 0.3, 9.1 ± 0.4, 8.6 ± 0.4 and 7.7 ± 0.3 ml/(g VSS·d) when the DCF level was 2.5, 7, 11.5, 25, 47.5 mg/kg TSS on 8 d. The results indicated that DCF could inhibit methane production during WAS anaerobic fermentation, and the inhibition gradually increased with DCF level increased from 2.5 to 47.5 mg/kg TSS, which was in good accordance with the previous literature (Symsaris et al., 2015).

3.3. COD mass balance analysis of WAS anaerobic fermentation at different DCF levels

In order to further understand the effect of DCF on VFA production, COD mass balance analysis was conducted by determining several typical intermediates or final products during WAS anaerobic fermentation, such as VSS, VFAs, soluble protein, soluble carbohydrate, hydrogen, and methane. The COD mass balance in these batch experiment reactors on 8 d is shown in Supplementary data. There is no distinct difference on COD percentages of soluble protein, soluble carbohydrate, and hydrogen among these reactors. However, the percentages of VSS, VFAs, and methane varied significantly with the variation of DCF level. For example, the percentage of methane decreased from 11.2 ± 0.4% to 8.6 ± 0.2% when DCF level increased from 2.5 to 47.5 mg/kg TSS, which indicated that the increase of DCF inhibited methanogenesis. As for VFAs, with the increase of DCF from 2.5 to 25 mg/kg TSS, the percentage increased from 3.9 ± 0.1% to 7.3 ± 0.2%, but

further increased DCF level to 47.5 mg/kg TSS, the percentage decreased to 5.9 ± 0.3%. Opposite trend was observed for VSS, the percentage of VSS decreased from 70.3 ± 1.9% to 66.3 ± 1.8% when DCF level increased from 2.5 to 25 mg/kg TSS, then increased to 67.9 ± 1.6% with DCF further increased to 47.5 mg/kg TSS. The results above indicated that with an increase of DCF level from 2.5 to 25 mg/kg TSS, more fermentation substrates were utilized for VFAs production and caused the reducing of VSS percentage, further increase of DCF level to 47.5 mg/kg TSS, however, inhibited VFAs production.

3.4. The degradation of DCF during WAS anaerobic fermentation

DCF can be degraded by the metabolic enzymes produced from microorganisms, and the main products were hydroxylation, sulfation, and grape glycosylated DCF (Osorio-Lozada et al., 2008; Shen et al., 1999). To further explore the interaction between DCF and WAS anaerobic fermentation, the degradation of DCF during the fermentation process was investigated. Fig.3 shows the variation of DCF's concentration in the 47.5 mg/kg TSS DCF reactor during WAS anaerobic fermentation in the initial 8 d. It can be seen that after fermentation for 6 d, the concentration of DCF was 8.0 mg/kg TSS, and then remained almost unchanged at the subsequent fermentation time, indicating that DCF was partly degraded in the WAS fermentation reactor and the remaining components of DCF were mostly refractory and stable.

3.5. Effect of DCF on each step involved in WAS anaerobic fermentation

Protein and carbohydrate, the main components of WAS, are normally in particulate state (Orr et al., 2016). The particulate substrates would be first transformed into the soluble ones for further utilization. Changes in soluble protein and carbohydrate can well represent the process of sludge disintegration. In this study, soluble protein and carbohydrate in the fermentation reactors were adopted to be the reasonable indicators to reflect the disintegration efficiency of WAS. As is shown in Fig.4a, there was no significant difference among these anaerobic reactors on the concentrations of soluble protein and carbohydrate after

fermentation for 2 days. The concentration of soluble protein was respectively 393, 404, 399, 415, and 401 mg COD/L in reactors with DCF dosage 2.5, 7, 11.5, 25, and 47.5 mg/kg TSS, and the corresponding concentration of soluble carbohydrate was 158, 154, 160, 162, and 157 mg COD/L. Similar experimental results were also found in other time (Data not presented). The result clearly showed that DCF gave no influence on the disintegration of WAS while it occurred in the fermentation systems.

Apart from the disintegration process, hydrolysis, acidification, acetogenesis and homoacetogenesis processes are also closely related to the accumulation of VFAs. In order to figure out how DCF affects these processes, a series of batch experiments were performed. As shown in Fig.4b, the degradation rates of BSA and dextran did not differ significantly among the reactors (p>0.05). For example, at the fermentation time of 3 d, the degradation rates of BSA and dextran in the 2.5 mg/kg TSS DCF reactor were respectively 19.5% and 30.4%, while in the presence of 25 mg/kg TSS DCF, the degradation rates of BSA and dextran were 19.8% and 29.7%, which were similar to the results of the former. The experimental results showed that DCF had no obvious effect on the hydrolysis process of WAS.

Fig.4c shows the effects of DCF on the acidification of hydrolyzed products (amino acid and monosaccharide) to VFAs on 3 d. With the concentration of DCF increased from 2.5 to 25 mg/kg TSS, the degradation efficiency of L-glutamate and glucose increased from 33.4% and 50.6% to 55.3% and 74.4% respectively, suggesting that proper increase in the concentration of DCF contributed to the acidification of macromolecules. However, when the concentration of DCF continued to increase to 47.5 mg/kg TSS, the degradation efficiency of L-glutamate and glucose decreased, as compared with that of 25 mg/kg TSS, indicating that high concentrations of DCF inhibited the acidification of macromolecules.

The appropriate concentration of DCF can significantly increase the content of VFAs, especially acetic acid (Fig.1), and acetic acid can be biotransformed by macromolecule organic degradation, acetogenesis of

macromolecule organic acid, or homoacetogenesis of hydrogen. From Fig.4c it can be seen that DCF has a role in promoting the degradation of macromolecules. One might want to know how the presence of DCF affects the acetogenesis and homoacetogenesis processes. In this study, the effect of DCF on the acetogenesis process was indicated by the degradation efficiency of sodium butyrate. It can be observed from Fig.4d that the degradation efficiency of sodium butyrate was obviously increased with the presence of DCF, which indicated that DCF stimulated the acetogenesis process. The degradation efficiency of sodium butyrate was respectively 11.2%, 12.4%, 18.9%, 25.1% and 22.7% when the dosages of DCF were 2.5, 7, 11.5, 25, and 47.5 mg/kg TSS on 4 d. The results indicated that the promotion of DCF on the acidification process was improved with the DCF dosage increased from 2.5 to 25 mg/kg TSS. However, while the DCF dosage further increased to 47.5 mg/kg TSS, this positive effect was decreased.

Meanwhile, acetic acid can be generated by homoacetogenic bacteria with hydrogen and carbon dioxide as substrates. In order to explore the mechanism about the enhancement of acetic acid production with the presence of DCF, it is necessary to investigate the effect of DCF on homoacetogenesis process. Fig.4e shows the hydrogen consumption and acetic acid production with different DCF levels on 3 d, it can be seen that both the hydrogen consumption and acetic acid production were increased while DCF occurred. It was reported that hydrogen can also be utilized for methane production, which may be one reason for the increase of hydrogen consumption (Wang et al., 2015b). The methane yield during this process with different DCF levels is shown in Supplementary data and no distinct difference can be observed. Thus it can be concluded that the homoacetogenesis process was promoted by DCF, and the promotion was improved when the DCF dosage increased from 2.5 to 25 mg/kg TSS. However, when the DCF dosage further increased to 47.5 mg/kg TSS, the promotion decreased.

Methanogenesis is the last biochemical process included in sludge anaerobic digestion, in which

methanogens consume acetic acid or hydrogen as substrates to produce methane. According to the results of H_2-CO_2 Test, the production of methane showed no distinct difference with different DCF dosages while hydrogen and carbon dioxide served as the substrates (Supplementary data). The results indicated that hydrogenotrophic methanogenesis was not affected by DCF. The effect of DCF on acetoclastic methanogenesis is also explored and the results are presented in Fig.4f. As shown in Fig.4f, with the increase of DCF concentration, the consumption of acetic acid decreased. For example, when the concentration of DCF increased from 2.5 to 47.5 mg/kg TSS, the consumption of sodium acetate decreases from 810 to 449 mg/L on 3 d. Meanwhile, with the increase of DCF concentration, methane production decreased from 53 to 29 mL. Similar experimental results were also observed at other fermentation time (data not shown). The above experimental results clearly suggested that DCF had a seriously inhibitory impact on acetoclastic methanogenesis, which can be served as a reasonable explanation for DCF inhibited methane yield during the fermentation process described in Section 3.2. DCF is a kind of toxic substance whose presence can inhibit the absorption and transformation of organic matter by microorganisms. It was reported that 5 μg/L DCF can damage the microbial cell walls in a SBR (Jiang et al., 2017). Methanogens are sensitive to the surrounding environment, thus the presence of toxic and hazardous substances would inhibit its metabolic process, resulting in the decline of methane production.

3.6 Effect of DCF on key enzyme activities related with acetic acid generation

Validation of key enzyme data shown in Fig.5 can reveal the effect of DCF on microbial metabolic processes. Protease, α-glucosidase, butyrate kinase, carbon monoxide dehydrogenase, and coenzyme F_{420} are the key enzymes responsible for protein hydrolysis, polysaccharide hydrolysis, butyric acid production, homoacetogenesis, and methane production, respectively, and acetate kinase as well as CoA-transferase are both responsible for acetic acid production. As shown in Fig.5, the presence of DCF had no significant impact

on the hydrolase (p>0.05), whereas significantly increases the activities of the acid producing enzymes. The activities of butyrate kinase, carbon monoxide dehydrogenase, acetate kinase and CoA-transferase enzymes in the 25 mg/kg TSS DCF reactor were respectively 1.09, 1.18, 1.39, and 1.21 times of that in the 2.5 mg/kg TSS DCF reactor. As for the F_{420}, DCF seriously inhibited the activity of F_{420}. All these observations are well consistent with the previous experimental results (Fig.4).

3.7 Effect of DCF on microbial community during WAS anaerobic fermentation

In order to further explore the reasons for DCF promoting acetic acid yield from sludge, the communities of anaerobic bacteria relevant to acetic acid production were conducted by Illumina HiSeq sequencing technique, and the results at phyla level are shown in Supplementary data. It can be seen that the microbial communities in both the 2.5 and 25 mg/kg TSS DCF reactors mainly consisted of three phyla: *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, all of them have been reported to possess the ability to degrade a wide range of complex organic matters for VFAs production (Leven et al., 2007; Wong et al., 2013; Zheng et al., 2013).

However, the abundance of these microbial species varied significantly between the 2.5 and 25 mg/kg TSS DCF reactors. *Firmicutes*, most of which were documented to possess the ability of consuming acetic acid (Ariesyady et al., 2007), was reduced when DCF level increased. The decrease of *Firmicutes* abundance in the 25 mg/kg TSS DCF reactor (40.8% in the 2.5 mg/kg TSS DCF reactor versus 32.3% in the 25 mg/kg TSS DCF reactor) was obviously advantageous to acetic acid accumulation. *Bacteroides*, which was reported to be in a position to produce acetic acid by degrading a variety of organic matters during sludge fermentation (Jang et al., 2014; Riviere et al., 2009), was increased with the increase of DCF level (12.5% in the 2.5 mg/kg TSS DCF reactor versus 16.4% in the 25 mg/kg TSS DCF reactor). *Proteobacteria* was studied to be a kind of anaerobic microorganism which generated acetic acid as major acidification products (Lu et al., 2012; Nelson

et al., 2011), and showed a higher abundance in the 25 mg/kg TSS DCF reactor (39.1% in the 25 mg/kg TSS DCF reactor versus 34.5% in the 2.5 mg/kg TSS DCF reactor).

Fig.6 further shows the shift of microbial abundances in genus-level while DCF occurred. It can be seen that several kinds of acetic acid - forming bacteria such as *Clostridium, Lactobacilus, Proteiniclasticum, Guggenheimella, Bacteroides, Paludibacter, Caldilinea, Collinsella, Acetoanaerobium*, and *Tissierella* were detected in both reactors. Except for *Acetoanaerobium*, the abundances of all other acetic acid producers were higher in the 25 mg/kg TSS DCF reactor compared with that in the 2.5 mg/kg TSS DCF reactor. For example, *Proteiniclasticum*, which was documented to possess the ability to utilize protein for acetic acid generation (Zhang et al., 2010; Zhao et al., 2017), was found to increase from 3.25% of the total bacterial sequences in the 2.5 mg/kg TSS DCF reactor to 4.36% in 25 mg/kg TSS DCF reactor. It was reported that *Clostridium* can produce acetate not only by degrading complex biopolymers, but also the conversion of hydrogen and carbon dioxide (Andreesen et al., 1970; Kamlage et al., 1997; Nelson et al., 2011), and was detected to be 8.21% and 8.87% in the 2.5 and 25 mg/kg TSS DCF reactors respectively. *Guggeheimella* was demonstrated to be able to produce several types of VFAs such as butyrate, valerate, propionate, and acetate under anaerobic conditions (Yuan et al., 2016), and its abundance was found to increase from 8.27% in the 2.5 mg/kg TSS DCF reactor to 9.35% in the 25 mg/kg TSS DCF reactor. According to above analyses, the microbial community in the 25 mg/kg TSS DCF reactor was more conducive to acetic acid production, which could be served as a possible reason for the former phenomenon (Fig.1).

4 Conclusion

It was found that DCF content was closely related to WAS anaerobic fermentation in this study. When the concentration of DCF increased from 2.5 to 25 mg/kg TSS, the maximum yield of VFAs increased from 599 to 1113 mg COD/L, but further increase of DCF caused decrease of VFAs, DCF mainly promoted the

production of acetic acid. The mechanism study showed that DCF had no obvious effect on sludge disintegration and hydrolysis and inhibited methanogenesis, but greatly improved the processes of acidification, acetogenesis, and homoacetogenesis. Microbial analysis showed that the presence of DCF could promote the enrichment of VFA producers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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Figure captions

Fig. 1. Effects of DCF on the accumulation of total VFAs with fermentation time (a), and the amount of individual VFA at their optimal fermentation time(b). Error bars represent standard deviations of triplicate tests.

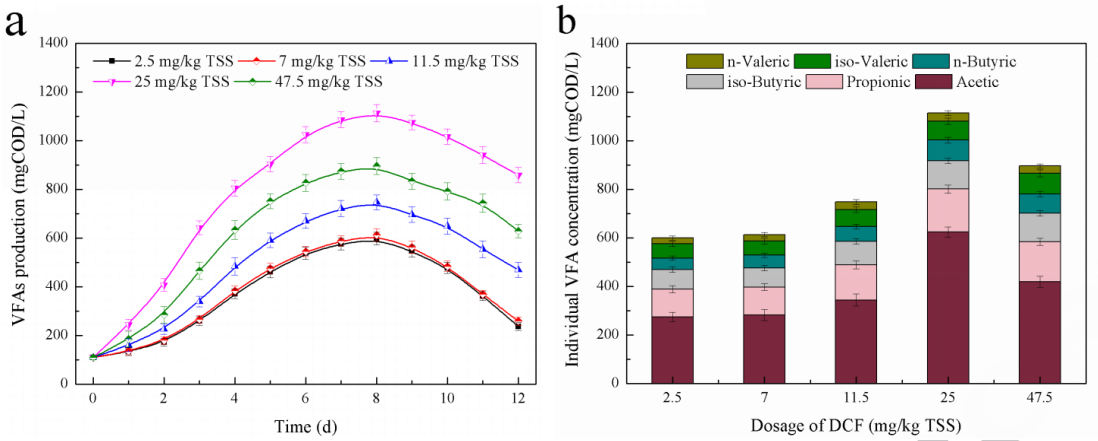
Fig. 2. Effect of DCF on the specific production rate of methane in the initial 8 days fermentation. Error bars represent standard deviations of triplicate tests.

Fig. 3. The variation of DCF's concentration during WAS anaerobic fermentation. Error bars represent standard deviations of triplicate tests.

Fig. 4. Effects of DCF on the disintegration (a), hydrolysis (b), acidification (c), acetogenesis (d), homoacetogenesis (e), and methanogenesis (f) processes of WAS anaerobic fermentation. Error bars represent standard deviations of triplicate tests.

Fig. 5. The effect of DCF on key enzyme activities. (BK: butyrate kinase; CODH: carbon monoxide dehydrogenase; AK: acetate kinase; F_{420}: coenzyme F_{420}). Error bars represent standard deviations of triplicate tests.

Fig. 6. The microbial abundances in the two long-term operated reactors (2.5 mg/kg TSS DCF reactor (a) and 25 mg/kg TSS DCF reactor (b)) in genus-level.



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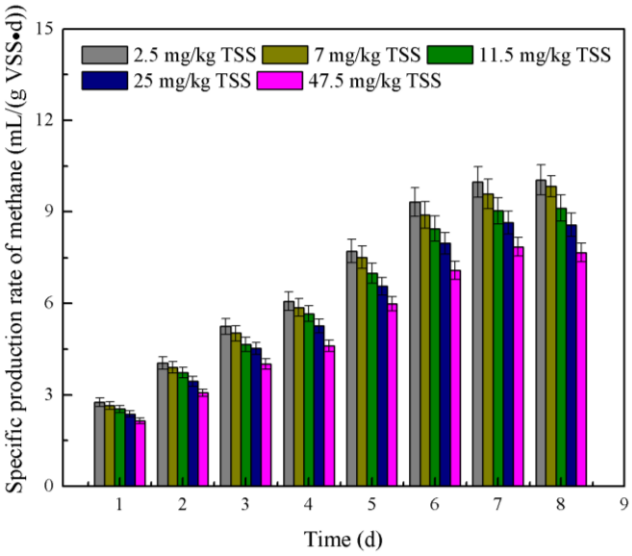


Fig.2. Effect of DCF on the specific production rate of methane in the initial 8 days fermentation. Error bars represent standard deviations of triplicate tests.

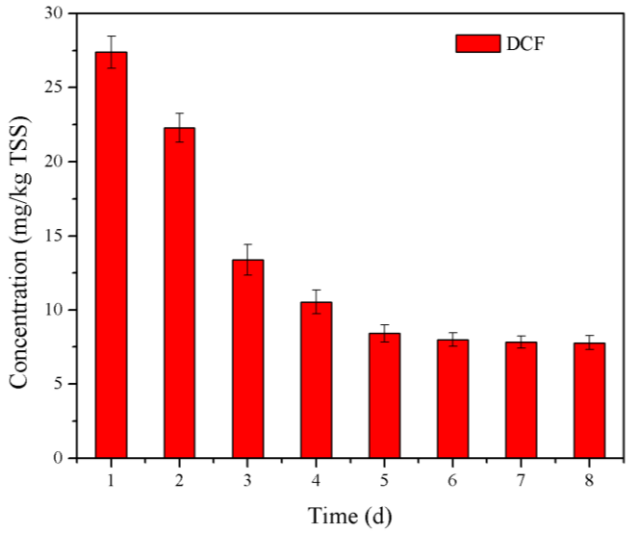
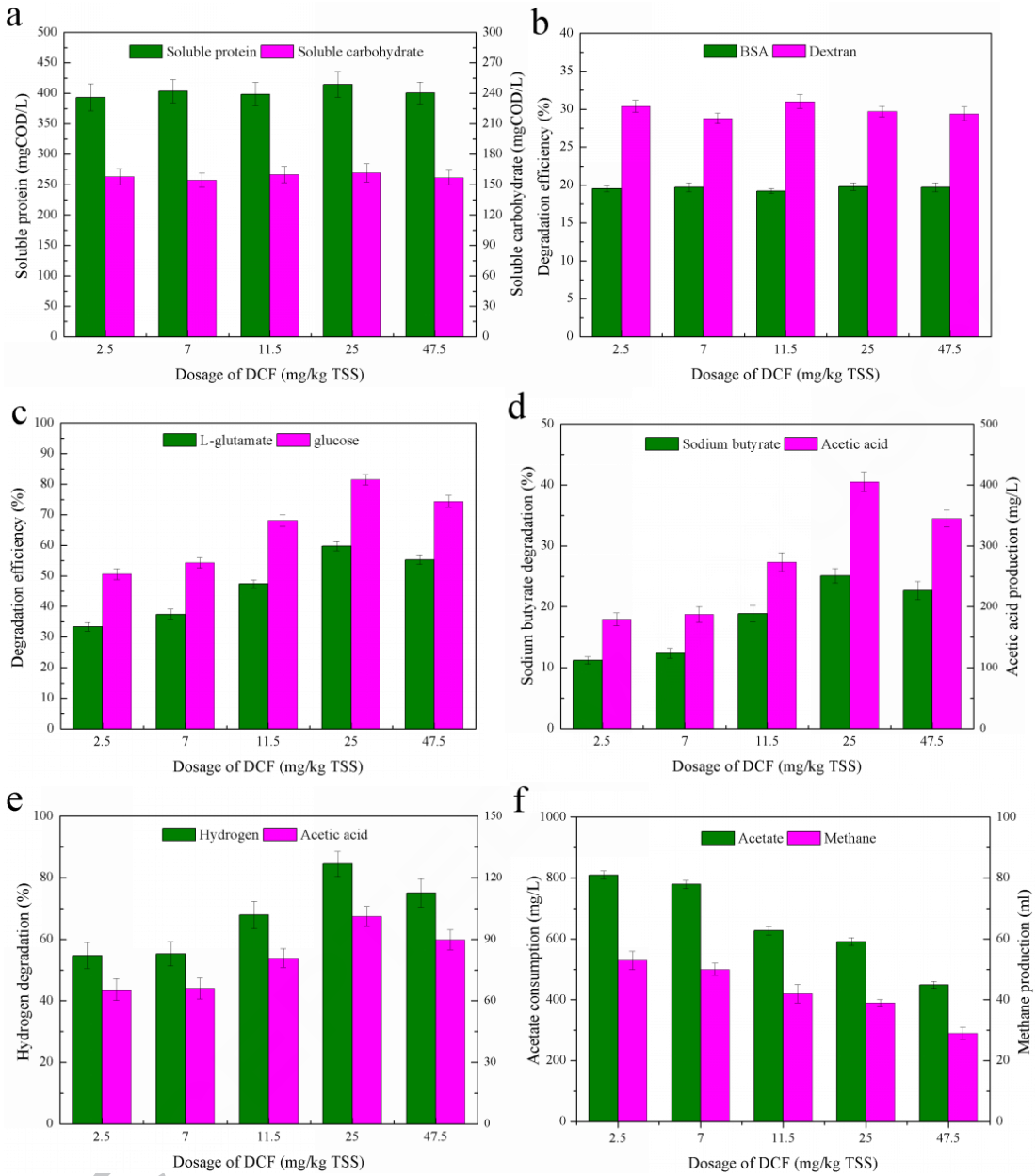


Fig. 3. The variation of DCF's concentration during WAS anaerobic fermentation. Error bars represent standard deviations of triplicate tests.



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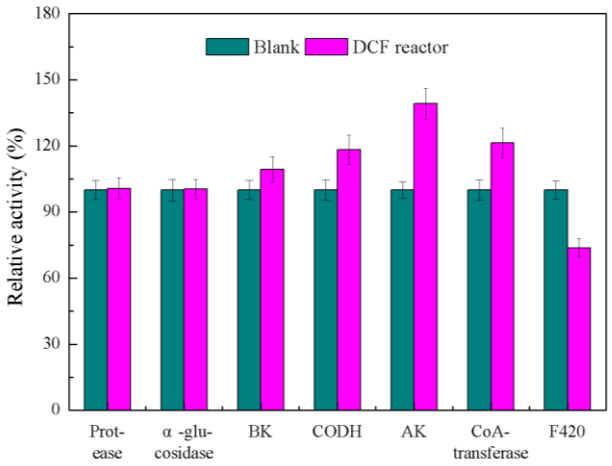

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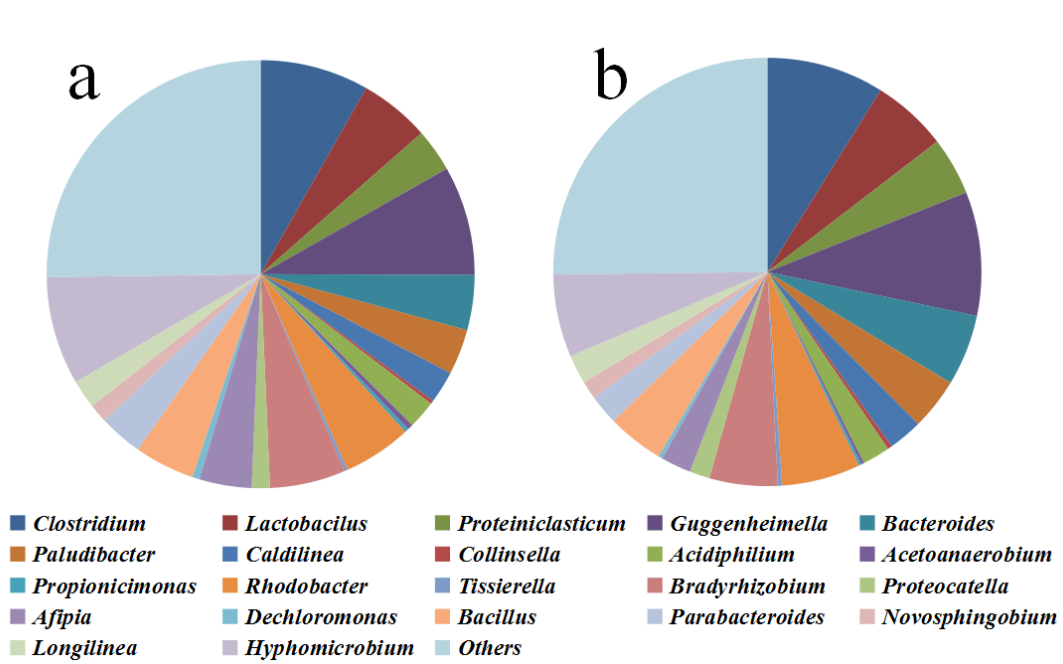

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Highlights:

- ► Diclofenac facilitated VFAs production from sludge anaerobic fermentation
- ► A significant level of diclofenac was degraded in the fermentation process
- ► Diclofenac promoted acidogenesis, acetogenesis, and homoacetogenesis processes
- ► Diclofenac inhibited methanogenesis process

Graphical Abstract