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Inhibition of tetracycline on anaerobic digestion of swine wastewater

Yiwei Liu [a,b,1], Xiang Li [a,b,1], Zhao Tan [a,b], Chunping Yang [a,b,c,*]

[a] College of Environmental Science and Engineering, Hunan University and Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha, Hunan 410082, China

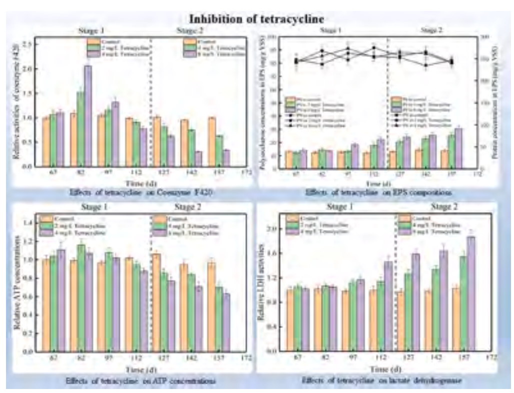
[b] Guangdong Provincial Key Laboratory of Petrochemical Pollution Processes and Control, School of Environmental Science and Engineering, Guangdong University of Petrochemical Technology, Maoming, Guangdong 525000, China

[c] Maoming Engineering Research Center for Organic Pollution Control, Academy of Environmental and Resource Sciences, Guangdong University of Petrochemical Technology, Maoming, Guangdong 525000, China

HIGHLIGHTS

- Tetracycline led to propionate accumulations.
- Tetracycline inhibited methanogenesis more heavily.
- The polysaccharide contents in EPS increased after exposure to tetracycline.
- Continuous tetracycline addition caused cells to be destroyed.

GRAPHICAL ABSTRACT



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ABSTRACT

The inhibition of tetracycline on anaerobic digestion of synthetic swine wastewater was examined with a semi-continuous operation for 103 days at a dosage ranging 2–8 mg/L. COD concentrations, VFA compositions in effluents and methane production were measured. The negative effects of tetracycline on the four individual steps of anaerobic digestion and its toxicity on anaerobic microorganisms were also evaluated. Results showed that continuous addition of 8 mg/L tetracycline in the bioreactor resulted in 73.28% reduction of daily methane production and made anaerobic digestion upset. Besides, methanogenesis was particularly inhibited compared to other three steps and the corresponding enzyme activities decreased by 66%. Furthermore, the polysaccharide contents in EPS increased after exposure to tetracycline, which could inhibit direct connections among micro-organism. At last, long-term exposure to tetracycline inhibit anaerobic microbial activities and caused liberation of lactate dehydrogenase. The results would provide novel insights for anaerobic digestion of swine wastewater.

* Corresponding author.
E-mail address: yangc@hnu.edu.cn (C. Yang).

[1] Both authors contributed equally to this work.

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1. Introduction

There is a growing demand on swine industry in China due to increasing human pork consumption (Chen et al., 2020b). Therefore, pollution caused by swine industry is inevitable as over 460 million tons of swine wastewater have been discharged each year (Xu et al., 2019). Direct discharge of swine wastewater which enrich of 3000–15000 mg/L chemical oxygen demand (COD), 400–1400 mg/L ammonia nitrogen (NH_4^+-N) and 100–250 mg/L total phosphorus (TP), and increases risks of eutrophication and black and odorous water (Cheng et al., 2018; Jiang et al., 2020; Li et al., 2020). These deleterious effects have caused severely threaten to human living environment in recent years.

Because of the high concentration of COD, anaerobic digestion (AD) is the predominant way to purify swine wastewaters because of the capacity of cost-effectively and energy recycle (Chen et al., 2020a; Huang et al., 2018). AD process was a promising biological treatment method, which was cooperation of bacteria and archaea in four sequential steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Gou et al., 2020). Failure to keep balance of bacteria and archaea or maintain stabilities of four steps might upset anaerobic digestion (Chen et al., 2008).

However, apart from ammonia nitrogen and total phosphorus, some poisonous antibiotics, such as tetracycline, also existed in swine wastewater (Zhou et al., 2021). Tetracycline was widely applied into animal feeds to maintain health and improve growth efficiencies due its broad spectrum of activity, high quality and low cost (Zhang et al., 2018b). Nevertheless, tetracycline or other antibiotics could not be completely absorbed by livestock and released by their metabolites or their original form into the environment through swine urine and feces (Hu et al., 2019). In swine wastewater, tetracycline was one of the most common found classes of antibiotics, with the concentration of 388.7 μg/L (Cheng et al., 2018). Other researchers found that the concentration of tetracycline reached up to 23 mg/kg in pig feces (Martinez-Carballo et al., 2007). Furthermore, the usage of antibiotics was predicted to continue to increase in the future (Liu et al., 2016). Thus, the concentrations of antibiotics discharged into environment would further increase.

Tetracycline was used to be against a range of organisms, such as Chlamydia, mycoplasma, rickettsia, protozoan, a number of gram-positive and gram-negative bacteria (Onal, 2011). The main inhibition effects of tetracycline were that it could inhibit bacterial protein synthesis by binding the 30S ribosomal subunit to prevent the association of the aminoacyl-tRNA to the ribosomal acceptor-A site and cause structural changes of 16S rRNA (Cetecioglu et al., 2013). Studies showed that tetracycline have detrimental effects on AD processes. For example, some researchers stated that 30% methane production reduced in anaerobic digestion with dosage of 9.8 mg/L tetracycline, while one research observed that same level inhibition was existed in higher concentration of tetracycline (28 mg/L) (Arikan, 2008; Stone et al., 2009). This difference was attributed to different inoculum and operation conditions. More specifically, Cetecioglu et al. (2013) claimed that tetracycline had lethal effects at 8.5 mg/L on anaerobic microbial community and stopped substrate utilization or methane production after long-time exposure to low dose tetracycline. Besides, they found that this situation could not be recovered after tetracycline dosing stopped. Tetracycline might also affect anaerobic metabolism. Cetecioglu et al. (2012) speculated that the possibility of a metabolic shift would appear under the condition of high dosage of tetracycline, which could affect *acetoclastic methanogens* and favor *homoacetogenic bacteria*. However, the mechanism of adverse effects of tetracycline on AD of swine wastewater, such as enzymatic reaction and poison points are still unknown. This is a bottleneck that restricts the development of AD for treatment of swine wastewater.

In this study, a synthetic swine wastewater was selected as digestion substrate aimed to find the inhibited effects of tetracycline on AD. The effects of tetracycline on anaerobic digestion performance of synthetic swine wastewater were evaluated. Meanwhile, the effects of tetracycline on each step of anaerobic digestion (hydrolysis, acidogenesis, acetogenesis and methanogenesis) and key enzymes on anaerobic digestion were examined. Furthermore, toxicity of tetracycline and microbial activities after long exposure for tetracycline were also studied. This work expected to provide some novel insights and data for anaerobic digestion of swine wastewater after long-term exposure to tetracycline.

2. Materials and methods

2.1. Inoculum source and antibiotics

Anaerobic digestion sludge was taken from septic tank in peasant household in Hunan province, which was never exposure for tetracycline. The septic tank was constructed for livestock wastewater and domestic water treatment, which was used for methane production for heating. Anaerobic digestion sludge was screened through 2 mm sieve to remove other impurities and stored at 4℃ before adding into reactors.

A kind of synthetic swine wastewater was used as influent of anaerobic reactors. The composition of synthetic swine wastewater was according to Cheng et al. (2020) and Bergmanna et al. (2000), which mainly contained 2500 mg/L COD (prepared by saccharose), 267.50 mg/L NH_4Cl and 83.25 mg/L Na_2HPO_4. All of these chemicals were analytical grade and gained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Tetracycline hydrochloride (purity 95–105%) was supplied by Hefei Bomei Biotechnology Incorporated Company (Hefei, China), which molecular formula was $C_{22}H_{24}N_2O_8 \cdot HCl$.

2.2. Experimental set-up

Three anaerobic reactors with 0.5 L total volume and 0.4 L working volume were set to evaluate disadvantages of tetracycline on anaerobic performance and microorganism. They were operated at 35℃ in shaking bath under dark conditions with rotate speed of 130 rpm. In anaerobic reactors, 100 mL anaerobic sludge in septic tank and 300 mL synthetic swine wastewater were added. The volatile suspended solid (VSS) was 6410 mg/L in the reactors. The reactors were operated a "semi-continuous" mode at a cycle of 24 h, which contained filling, reaction and drawing. pH was adjusted after drawing and filling with 2 g/L $NaHCO_3$. Daily methane production and effluent COD concentration were measured.

Before tetracycline addition, the reactors operated with a lower organic loading rate and increased organic loading rate in a stepwise manner. Firstly, the reactors operated at 1000 mg/L COD. Once COD removal efficiency was higher than 80% and methane production kept stable in each reactor, influent COD was climbed up to 2000 mg/L. Eventually, the reactors were increased to 2500 mg/L influent COD with the same way. Under 2500 mg/L COD influent, the concentrations of volatile fatty acids (VFAs) in effluent were measured. When COD concentrations, volatile fatty acids in effluent and daily methane production remained stable, tetracycline was added. The whole experiments divided into two stage. During the first stage, the two experimental reactors were dosage with 2, 4 mg/L tetracycline for 54 days. In the second stage, the concentrations of tetracycline increased to 4, 8 mg/L in these two reactors, respectively.

Methane production, VFAs and effluent COD was monitored every day. Key enzymes in anaerobic digestion were also measured in a 15-day interval, such as dehydrogenase (DHA), acetate kinase (AK), and coenzyme F_{420}. To evaluate the toxicity of tetracycline on anaerobic microorganism in semi-continuous mode, the concentrations of adenosine-triphosphate (ATP), activities of lactate dehydrogenase (LDH) and extracellular polymeric substance (EPS) were also measured at the same intervals.

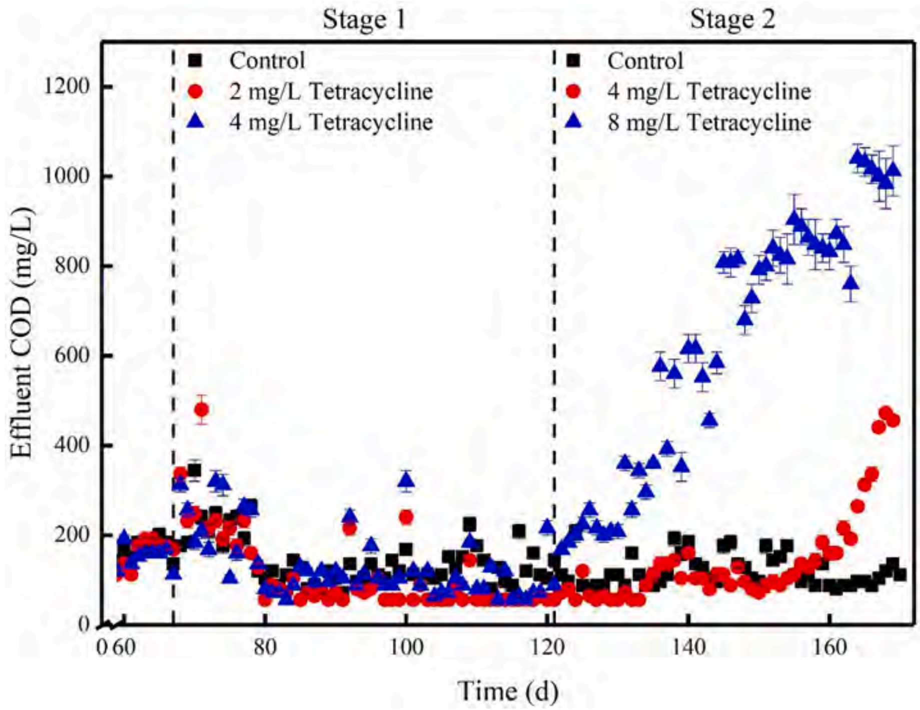

Fig. 1. Dynamic changes of COD in effluents among three reactors with different tetracycline concentrations.

2.3. Batch assays

In order to evaluate adverse effects on each step of anaerobic digestion. Four batch tests were carried out with different carbon resources corresponding to hydrolysis, acidogenesis, acetogenesis and methanogenesis. Anaerobic sludge was taken out when anaerobic performance presented significant difference in each reactor.

Test 1: Test 1 was designed to investigate the impacts of tetracycline on hydrolysis during anaerobic digestion of swine wastewater. In the batch test 1, three groups of serum bottles with 90 mL synthetic swine wastewater and 10 mL anaerobic sludge obtained from anaerobic reactors mentioned above. The synthetic swine wastewater contained 0.39 g/L bovine serum albumin (BSA, average molecular weight of 67,000, a model protein compound) and 0.6 g/L dextran (average molecular weight of 40,000, a model polysaccharide compound) as carbon resources, respectively. Other elements in synthetic swine wastewater were consistent with those mentioned above. Different amount of tetracycline was also added into each bottle to reach levels of 0, 4, 8 mg/L. These serum bottles were set in shaking bath with 35℃. After reaction, the synthetic swine wastewater was fetched to monitor concentrations of protein in three reactors to evaluation their degradation rates at 1, 2, 4, 12, 20 h. The concentrations of saccharides were monitored at 1, 4, 10,16,24, 32, 48 h.

Test 2: This series batch experiment was set to investigate the impacts of tetracycline on acidogenesis step in anaerobic digestion of swine wastewater. Apart from carbon resource, other reaction conditions were as same as the Test 1 above. In this batch reaction, glucose was used to be carbon resource. The whole experiment lasted at 24 h and the concentrations of glucose were monitored at 1, 2, 4, 10, 16, 24 h.

Test 3: The batch test 3 was aimed to evaluate the inhibition effects of tetracycline on acetogenesis process in anaerobic digestion of swine wastewater. The operation was the same as batch Test 1 except that the carbon resources were replaced by propionate. The concentrations of propionate in three reactors were monitored at 12, 24, 36, 48, 60, 72 h.

Test 4: This batch experiment was carried to evaluate the inhibition effects of tetracycline on methanogenesis step in anaerobic digestion of swine wastewater. The experiments were set in a series of serum bottles with a volume of 250 mL. Each bottle contained 40 mL anaerobic sludge from semi-continuous reactors and 160 mL synthetic swine wastewater. The carbon resource of this experiment was only 2 g/L sodium acetate, and other conditions were same as the Test 1 mentioned above. This batch experiment lasted 6 days. The methane production in each bottle was monitored at 24-hour interval. Then cumulative methane production was calculated.

2.4. Analytic methods

COD, VSS was performed according to standard methods (APHA, 2012). Polysaccharide and protein were determined by the anthrone-sulfuric method with glucose as the standard and the Lowry-Folin method with BSA as the standard, respectively (Tang et al., 2020; Zhao et al., 2015). Biogas volume measurement measured by water replaced method. Methane fraction in gas samples was determined by use of a gastight syringe to inject 0.2 mL of the samples into a gas chromatograph (GC112A, China) (Wang et al., 2015). The detections of VFA and specific procedures were according to previous study (Wang et al., 2017). As for key enzyme, 1 g diluted sludge mixture were taken out, added by 5 mL buffer solution, then ultrasonically broken at 20 kHa at 0 ℃ for 5 min and finally centrifuged at 10000 rpm at 4℃ for 10 min. The extracts were placed in ice-water bath before measurement. The measurements for the activities of DHA, acetate kinase and coenzyme F_{420} were according to previous studies (Chen et al., 2018; Wang et al., 2015). At the same time, the contents of ATP and activities of LDH were also tested. Specific test methods were based on the assay kit purchased by Nanjing Jiancheng and use according to manufacturer's instructions. The extraction of EPS was according to previous study (Tang et al., 2020).

2.5. Statistical analysis

Origin 2017 (OriginLab Corporation, USA) was used for the construction of all figures in this work. All tests were conducted in triplicate. An analysis of variance was used to evaluate the significance of results, and $p < 0.05$ was considered to be statistically significant.

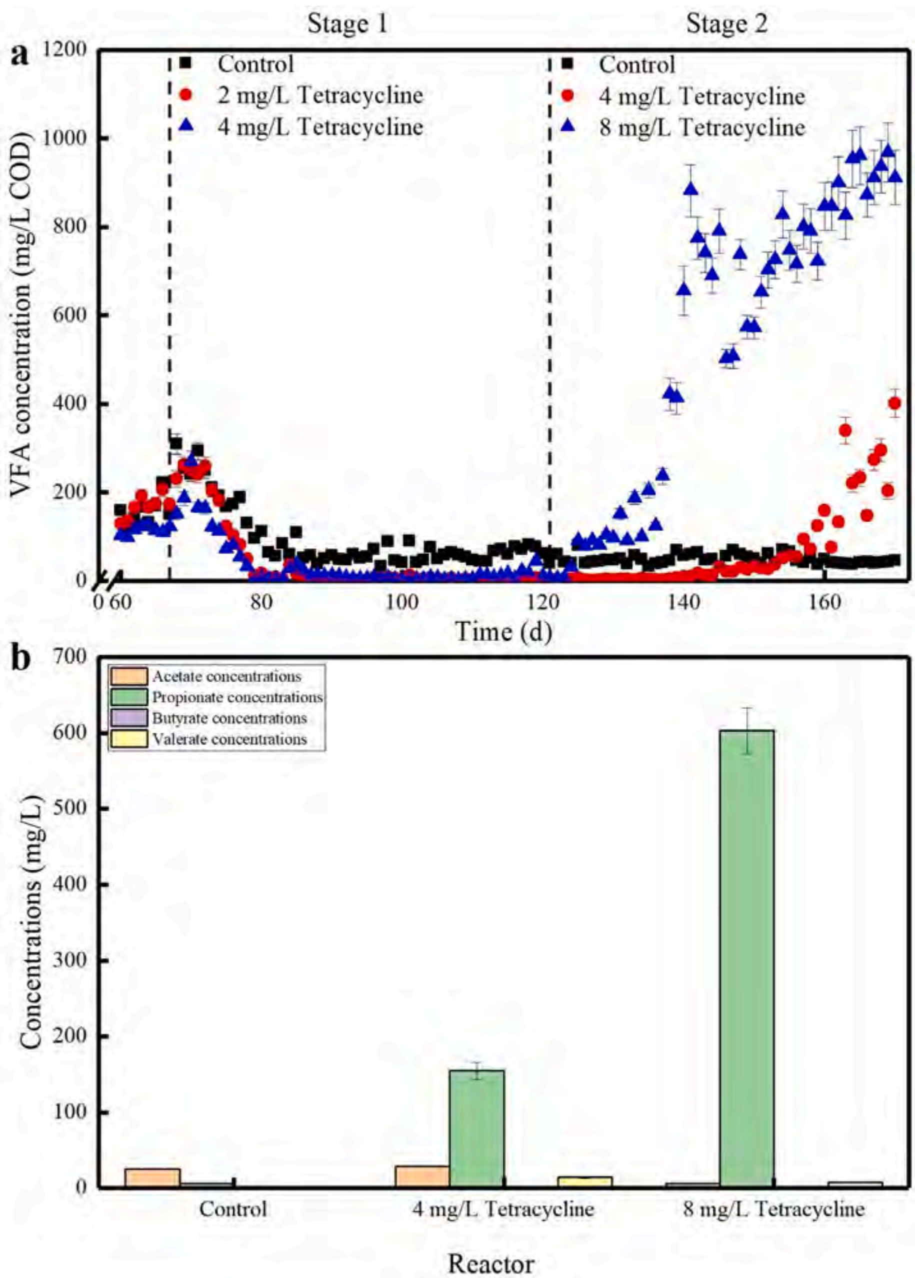

Fig. 2. (a) Dynamic changes of VFA concentrations in effluents among three reactors; (b) The VFA compositions in effluents of three reactors in 168th day.

3. Results and discussion

3.1. Effects on anaerobic digestion performance

3.1.1. Effects on COD removal

According to Fig. 1, efficient COD remove could be observed after a 66-day acclimation. Effluent COD in three reactors were reduced from influent COD concentration of 2500 mg/L at the beginning to 184 mg/L, with corresponding to COD removal efficiencies higher than 92%. The control reactor maintained similar COD removal efficiency for the entire monitoring time, which could illustrate that synthetic substrate could be completely degraded and utilized by anaerobic microorganism at the beginning. Residual COD mainly contained VFAs and soluble microbial products according to others' researches (Amin et al., 2006).

As illustrated in Fig. 1, the addition of 2, 4 mg/L tetracycline had no noticeable inhibition effects on COD remove in semi-continuous anaerobic digestion of swine wastewater. The effluent COD concentrations in experimental reactors decreased slightly compared to the control, which were both lower than 100 mg/L in initial tetracycline dosing. Cetecioglu et al. (2013) also observed this phenomenon that effluent COD concentrations were slightly lower than that of control after initial exposure for tetracycline. Furthermore, COD removal efficiencies kept stable in stage 1, in which tetracycline was added with 2, 4 mg/L. In stage 2, tetracycline concentrations added in two experimental reactors was further increased. Once rising tetracycline concentrations, anaerobic digestion performance was upset. The effluent COD value in 8 mg/L tetracycline reactor appeared fluctuations and then increased stepwise to more than 1000 mg/L after 160th day. As for low dosage of tetracycline reactor, COD removal efficiency kept stable until 130 days, then it fluctuated in the following 30 days and finally effluent COD concentration increased. The effects of tetracycline on anaerobic digestion performance depended on its concentrations, which followed "dose–response" relationships. This phenomenon might be resulted from tetracycline behaviors in anaerobic digestion. Cheng et al. (2020)

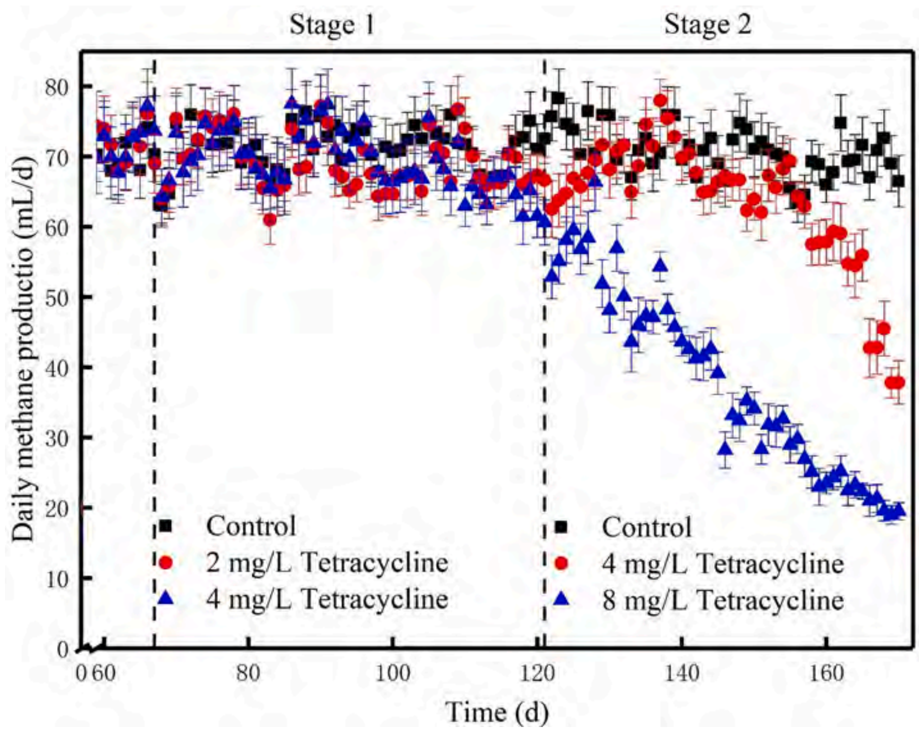

Fig. 3. Effects of different tetracycline concentrations on daily methane production.

confirmed that behaviors of tetracycline in anaerobic digestion was mainly biosorption. Thus, in the initial addition of tetracycline, it could be adsorbed and then reached saturation sorption to inhibit microbial activities.

3.1.2. Effects on effluent VFA composition

The concentrations and compositions of VFAs in the effluent were monitored every day for providing more details about effects of tetracycline on anaerobic digestion of swine wastewater. Before addition of tetracycline, VFA concentration of each reactor maintained a low level according to Fig. 2a. The concentration of the control reactor fluctuated around 100 mg COD/L all the experimental time. Interestingly, the effluent VFA concentrations of other two experimental reactors decreased and were lower than that of the control, which were consistent with effluent COD. With the operation of reactors and continuous addition of tetracycline, the concentrations of VFAs in two experimental reactors appeared VFA accumulation, which eventually reached up to 401.56 mg COD/L and 911.27 mg COD/L, respectively. Aydin et al. (2015c) also observed that VFAs were accumulated after exposure for antibiotics. As illustrated in Fig. 2b, the composition of three reactors presented huge differences. In the control reactor, the concentrations of acetate, propionate, butyrate and valerate decreased successively, while in two experimental reactors, propionate was accumulated, which achieved 154.72 mg/L and 603.00 mg/L in two experimental reactors in day 168.

Long-term exposure for tetracycline resulted in VFA accumulation, especially propionate accumulation. VFA accumulations had negative effects on anaerobic digestion (Abubackar et al., 2019). They could inhibit methanogenic activities and lead to methane production decreased (Beneragama et al., 2013; Fang et al., 2020). More specifically, propionate accumulation might illustrate that the activities of propionate-oxidizing bacteria were inhibited. A previous study also speculated that propionate- and butyrate-oxidizing bacteria were inhibited due to their reduced methanogenic activities (Aydin et al., 2015a). Besides, propionate accumulations might inhibit subsequent processes of anaerobic digestion. One research concluded that propionate was the toughest to be utilized by syntrophic acetogenic bacteria among VFAs (Zhang et al., 2018a). In anaerobic digestion, propionate degradation was thought to be thermodynamically challenging due to its higher Gibbs free energy compared to the other VFAs (Gou et al., 2020; Mueller et al., 2010). Propionate accumulation might inhibit the conversion to acetate, which could decrease the speed of methane production.

3.1.3. Effects on daily methane production

Methane production is the result of COD conversion. It is the last step of anaerobic digestion and could be used to evaluate the magnitude of anaerobic microbial activities (Lu et al., 2016). During the initial operation time without tetracycline addition, methane productions of three reactors were around 72.94 mL/day, illustrating that the specific methane production was 0.21 L/g COD removed. This value was lower than other's researches due to different inoculated sludge, elements of digestion substrate and reactor types (Aydin et al., 2015b).

Fig. 3 showed daily methane yield in the three reactors during the entire experiment time. As the control, daily methane production in the control was stable and fluctuated around 72.94 mL/d for the whole monitoring time, which could state that the control reactor was in a stable state. As for other two experimental reactors, low tetracycline-dosing had no significant effects on methane production at initial, and even stimulate methane production. The daily methane production in experimental reactors kept in the same pace with the control around 72.94 mL/d. However, daily methane yield dropped gradually during the stage 2, when the concentration of tetracycline climbed up to 8 mg/L. Until the last day of experiment, daily methane production reached lowest, which was only 19.49 mL/d. Furthermore, specific methane production was only 0.08 L/g COD removed. Daily methane yield in the reactor with 4 mg/L tetracycline kept stable in a longer period compared to the reactor of 8 mg/L tetracycline. However, after the 150th day, daily methane production decreased dramatically, which was earlier than inhibition of COD removal efficiency.

Thus, the effects of tetracycline on methane production mainly included lower methane production and specific methane production. It was widely reported that tetracyclines had inhibition effects on methane production. For example, one research concluded that chlortetracycline

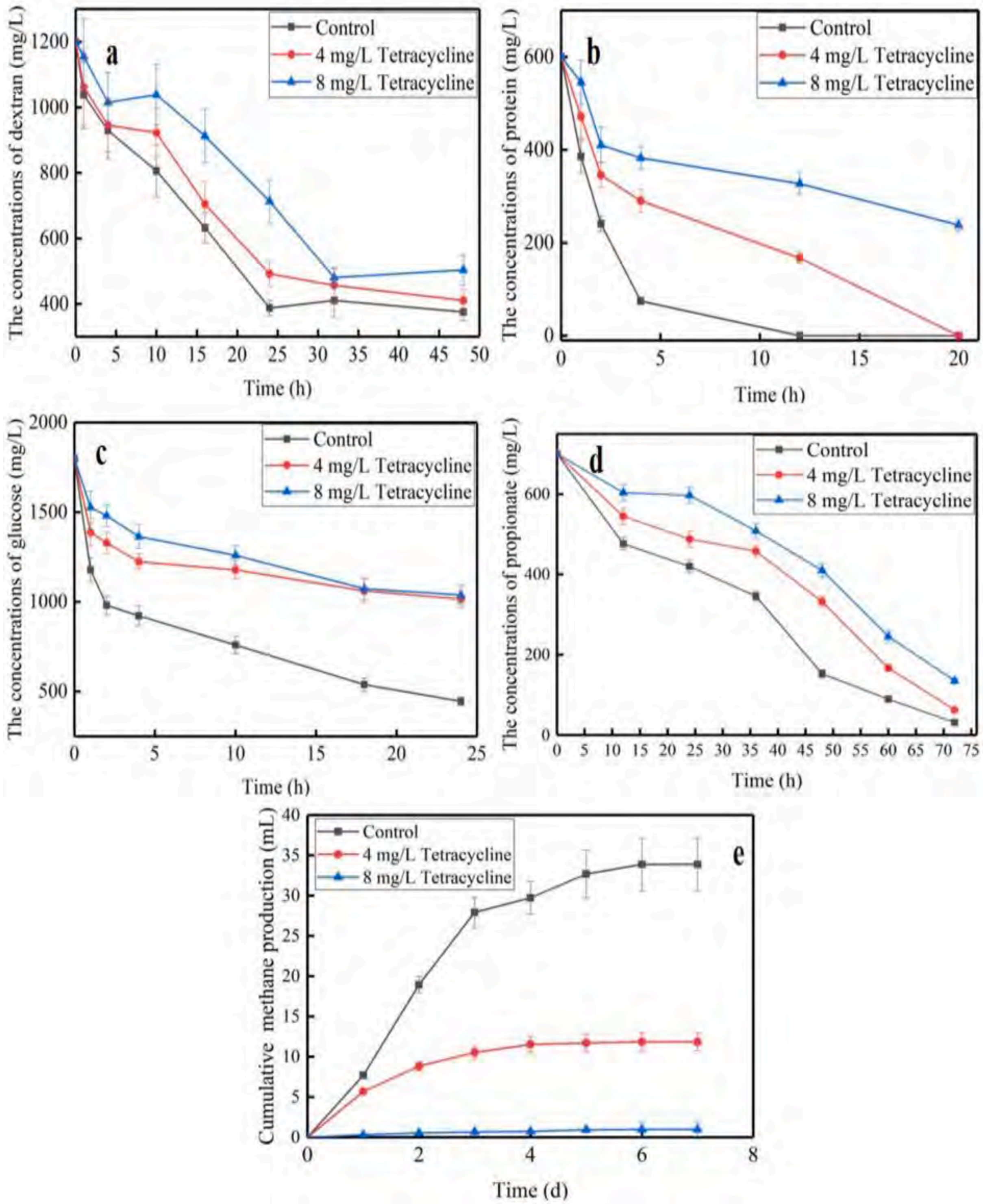

Fig. 4. (a) Effects of tetracycline addition on dextran degradation; (b) Effects of tetracycline addition on bovine serum albumin degradation; (c) Effects of tetracycline addition on glucose degradation; (d) Effects of tetracycline addition on propionate conversion; (e) Effects of tetracycline addition on acetate utilization.

could inhibit methane production rates and result in 27.8% reduction of total methane production (Stone et al., 2009). More specifically, Sanz et al. (1996) found that doxycycline could inhibit activities of acetogenic bacteria to result in 25%-45% reduction in methane production in the dosage of 10–100 mg/L. Lower specific methane production meant lower efficiencies of removed COD converting to methane. According to Xiong et al. (2017), there existed three main pathways for COD remove, which were converting to biomass, methane and remaining in effluents. Lower efficiencies of converting COD to methane could increase other two COD removal pathways, which illustrated that the addition of tetracycline could change substrate utilization modes of anaerobic microorganism. Besides, other researchers also stated at lower tetracycline concentrations, substrate utilization was not affected and COD removal efficiencies were similar with the control but methane production reduced. They thought it was stoichiometric disturbance, which was resulted from blockage of certain enzymatic steps in related

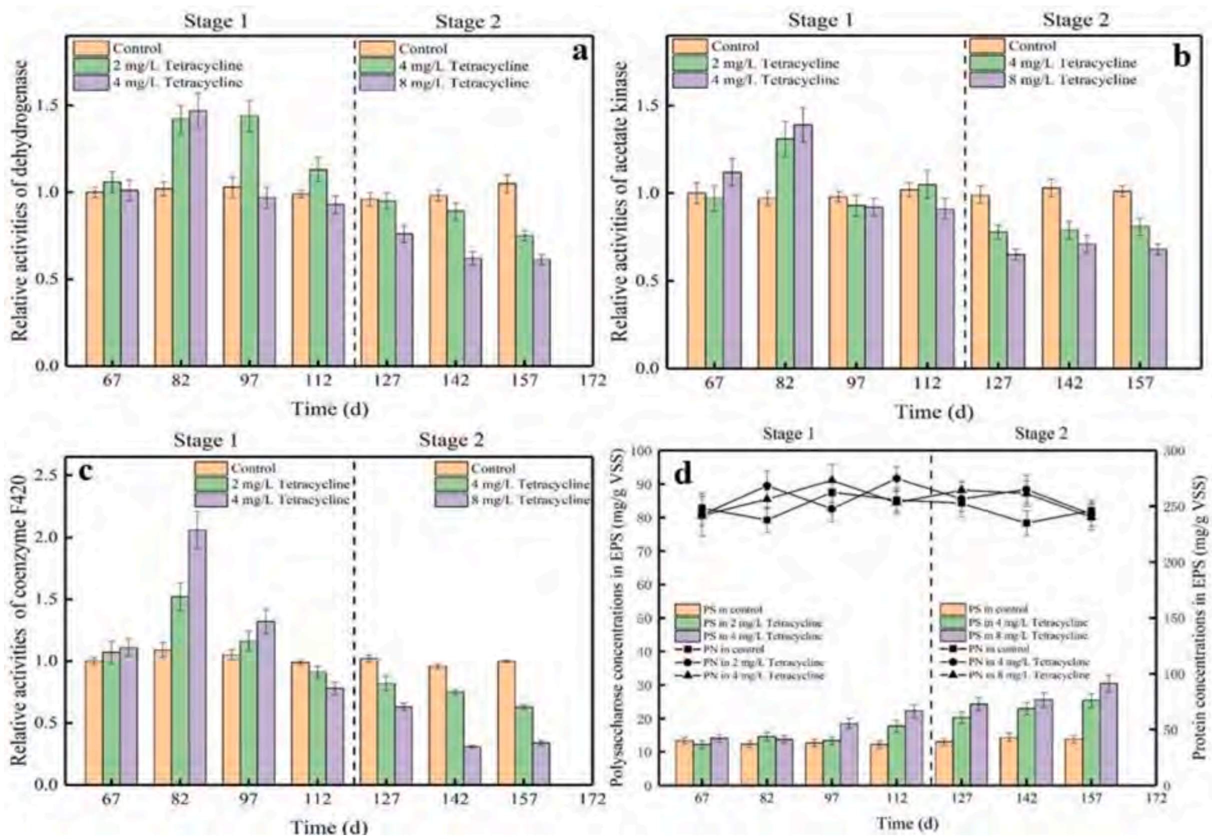

Fig. 5. Effects of tetracycline on (a) the relative activities of DHA; (b) the relative activities of AK; (c) the relative activities of coenzyme F_{420}; (d) EPS contents.

biochemical reactions. Besides, the authors illustrated that this phenomenon was attributed to substrate binding effects of tetracycline, similar to uncompetitive inhibition analogy (Cetecioglu et al., 2013; Fountoulakis et al., 2008).

3.2. Effects on anaerobic metabolism and activities

3.2.1. Effects on four individual steps of anaerobic digestion

Fig. 4 presented the effects of tetracycline on four steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) of anaerobic digestion. In five batch experiments, different carbon sources were selected which were corresponding to four steps of anaerobic digestion. According to Fig. 4a and Fig. 4b, tetracycline had an inhibitory effect on hydrolysis of anaerobic digestion. Protein could be completely degraded in the control and the reactor with the addition of 4 mg/L tetracycline at 20 h, while with the addition of 8 mg/L tetracycline, the degradation speed of protein decreased and 237.84 mg/L protein could not be hydrolyzed at 20 h. However, the degradations of dextran did not present same apparent differences among three reactors. This phenomenon was that anaerobic microorganism was acclimated under the condition of saccharose as carbon sources in semi-continuous operation. Similarly, as illustrated in Fig. 4c, the addition of tetracycline inhibited acidogenesis of anaerobic digestion. There was a significant difference of the consumption rates of glucose between the control and the experiment. Interestingly, eventually residual glucose concentrations were less affected by tetracycline concentrations in two experiments.

It can be seen from Fig. 4d and Fig. 4e that tetracycline inhibited acetogenesis and methanogenesis processes in anaerobic digestion of swine wastewater. At initial 48 h, the propionate in each group were consumed by 78.21%, 52.53% and 41.39%. The degradation of propionate was obviously inhibited with the addition of tetracycline. Besides, the degradation rate of propionate was correlated with tetracycline concentration. Afterwards, the propionate concentrations were nearly completely degraded in the control group and the group with 4 mg/L tetracycline, which were only 31.12 mg/L and 62.46 mg/L at last, respectively. While in the group with the addition of 8 mg/L tetracycline, the propionate concentration was 135.38 mg/L. At last, the accumulative methane production profile also presented inhibitory effects of tetracycline on methanogenesis step. Not only did tetracycline inhibited the rate of methane production, but it decreased the total methane production in this batch experiments. In the first 4 days, the methane was rapidly produced in the control group and the group with dosage of 4 mg/L tetracycline while in the group with addition of 8 mg/L, there was a little methane produced. The rate of methane production was decreased by 61.24% (from 7.43 mL/d to 2.88 mL/d) during the first 4 days. When the level of tetracycline further increased, the rate of methane production decreased more dramatically, which dropped by 97.58%. The inhibitory effects of tetracycline on methanogenesis processes would be enhanced with increasing tetracycline.

Thus, the individual steps of anaerobic digestion tests illustrated that tetracycline could inhibit methane production due to its negative effects on hydrolysis, acidogenesis, acetogenesis and methanogenesis, which was far different with the effects of roxithromycin on waste sludge anaerobic digestion (Ni et al., 2020). This phenomenon was mainly due to different substrates, inoculum and properties of antibiotics.

3.2.2. Effects on key enzymes

Anaerobic digestion is a bioprocess, during which the reactions are catalyzed by various enzymes. To further investigate metabolism of dynamic change after exposure for tetracycline, the activities of DHA, AK and coenzyme F_{420} were measured at 15-day interval. All of them are related to anaerobic digestion (Liang et al., 2020; Yang et al., 2019). DHA could reflect the degrading ability of pollutants and organics (Chen et al., 2018). AK and coenzyme F_{420} are corresponding to acidification and methanogenesis steps, respectively (Zou et al., 2021).

It was clear from Fig. 5a-c that the activities of three enzymes were

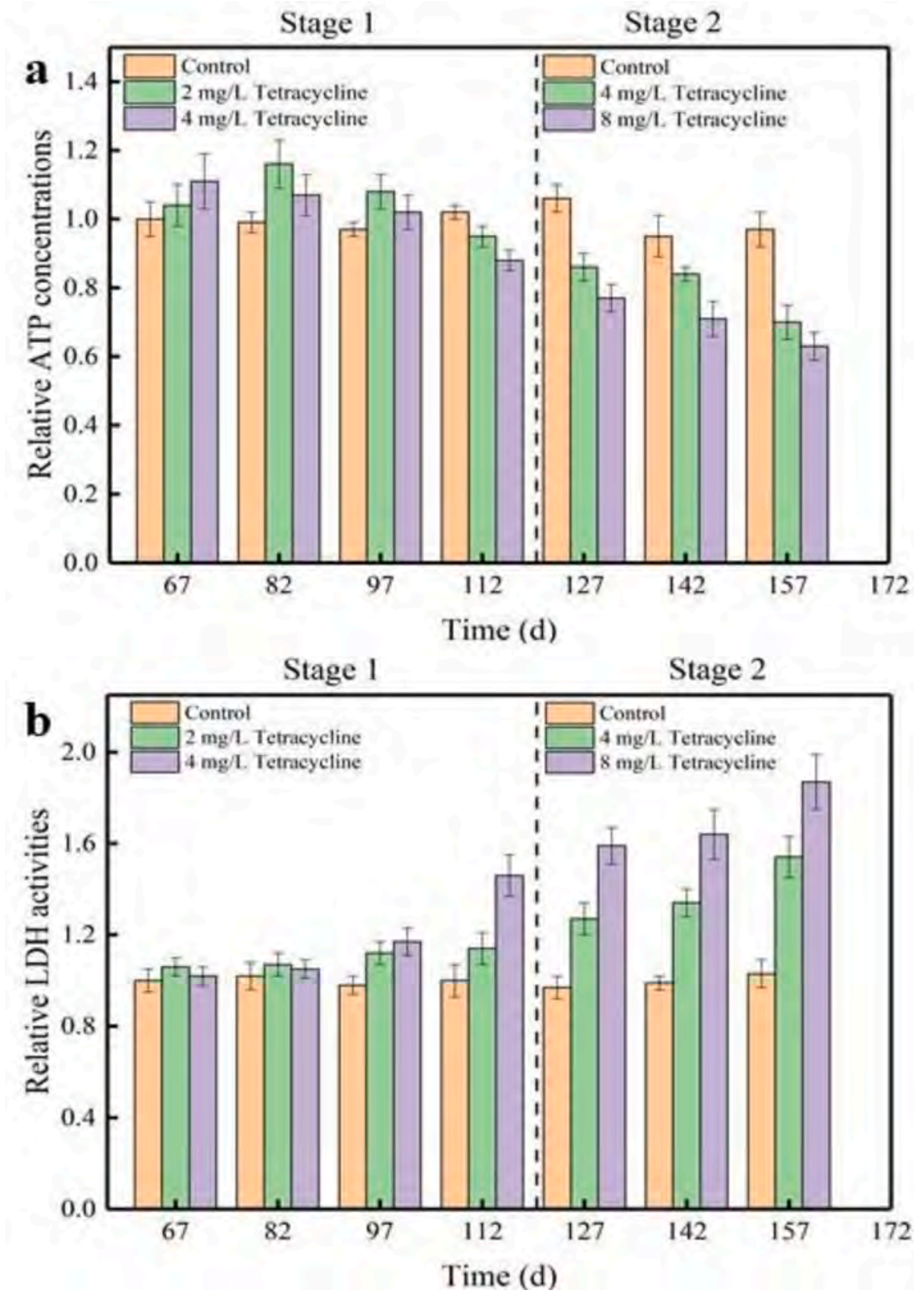

Fig. 6. (a) Effects of tetracycline on ATP concentrations; (b) Effects of tetracycline on LDH release.

decreased with the addition of tetracycline and the inhibition effects had positive relationships with dosage of tetracycline at the end of the experiment. At initial exposure for tetracycline, three enzyme activities increased in two experimental reactors. The highest enzyme activities were attained in the 15th day after addition of tetracycline. The higher enzyme activities could improve anaerobic digestion, which were consistent with slightly enhanced anaerobic digestion performance at beginning. Xie et al. (2019) also found that after 60-day exposure for antibiotics, the activities of enzymes improved. With operation of three reactors, all enzyme activities decreased. For DHA and AK, the relative activities in two experiment reactors were only 0.81, 0.68 and 0.75, 0.61 compared to the control. Furthermore, the activity of coenzyme F_{420} was inhibited more seriously and the relative activities in two experimental reactors were only 0.35 and 0.34. Thus, according to enzyme activities in each group, the continuous addition of tetracycline could inhibit each steps of AD and the methanogenesis step was more sensitive to tetracycline, which was in harmony with the batch experiments mentioned above.

3.3. Effects on anaerobic microbial properties

3.3.1. Effects on EPS contents

EPS was produced in microbial metabolism and its main components were protein (PN) and polysaccharides (PS), which accounted for 70%-80% (Chen et al., 2018). In this study, PN and PS were measured. As shown in the Fig. 5d, PN concentrations in EPS of three reactor presented no significantly differences and fluctuated around 250 mg/g VSS. This phenomenon could illustrate that the addition of tetracycline had no effects on PN in EPS. However, as for PS, their concentrations in two experimental reactors increased gradually after tetracycline addition. PS concentrations in three reactors were 13.32, 12.16 and 14.04 mg/g VSS at initial. After 90-day exposure for tetracycline, the highest PS concentration in EPS was attained in the highest concentration tetracycline reactor, which was 30.41 mg/g VSS and was nearly three times as high as the control. In low tetracycline-dosage reactor, PS concentration in EPS was slightly lower than that in 8 mg/L tetracycline and its concentration was 25.39 mg/g VSS, which was also far higher than that of the control reactor.

Increased EPS production was thought to be a response of anaerobic microorganism to protect themselves against external environmental stress (Shi et al., 2017). Furthermore, PS played major roles among various components of EPS and was thought to be fine strands attached on microbial cell surface, which could form networks to protect themselves (Yan et al., 2019). In this study, the tetracycline addition had positive relationships with EPS secretion, especially PS contents, which could adequately illustrate tetracycline toxicity on anaerobic microorganism. Increased PS secretion was proven to inhibited anaerobic performance. Li et al. (2019) stated that complex networks formed by PS might decreased the possibility of direct contact between reactive surfaces of cells, which was unlikely beneficial for the efficiency of mass transfer.

3.3.2. Effects on ATP contents and LDH release

ATP, as a bioenergy source in the microbial metabolism reaction, could be viewed as an indicator for microbial activities (Wang et al., 2016). The change of ATP concentrations during the whole experiment was presented in Fig. 6a. At initial 30-day tetracycline addition, ATP contents would increase compared to the control. With the persistent tetracycline addition, ATP concentrations in two tetracycline-amended reactors were affected by tetracycline and their concentrations decreased by 30% and 37% compared to the control eventually. Declined ATP concentrations illustrated that the activities of anaerobic microorganism were inhibited after long-term exposure for tetracycline, which might be a reason for less methane production. Xiong et al. (2017) concluded that dosage of 20 mg/L tetracycline caused ATP concentration decreases and low tetracycline level had no significantly influences on ATP concentrations.

LDH release was used to evaluate tetracycline toxicity on anaerobic microorganism. LDH presented in various microorganism and its release usually illustrated that the cell membrane permeability changed or the cell was destroyed (Tang et al., 2021). As shown in Fig. 6b, the current experiment found that LDH was increasingly released with the addition of tetracycline and its release was affected by tetracycline concentrations. At Stage 1, with the low-level addition of tetracycline, LDH release presented no remarkable differences. When tetracycline persistently added, LDH release increased sharply. The eventually relative LDH release in experimental reactors were 1.54 and 1.87 compared to the control. One research confirmed that azithromycin, a kind of macrolide antibiotic, also could cause LDH release in anaerobic sludge and authors claimed that these biochemical indicators, such as LDH release, could connect with anaerobic performance (Liu et al., 2018). Thus, the tetracycline toxicity might destroy cell integrity and then influence their performance.

4. Conclusions

The presence of tetracycline in swine wastewater significantly inhibited anaerobic digestion performance, such as lower COD removal, VFA accumulation and lesser methane production. Short-term and low tetracycline dosage might stimulate anaerobic microorganism. The inhibition effects would appear after long-term exposure. Moreover, tetracycline had negative effects on four individual steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The activities of key enzymes (DHA, AK and coenzyme F_{420}) during these steps also decreased. Furthermore, the polysaccharide contents in EPS increased after exposure to tetracycline and the tetracycline addition decreased microbial activities and caused the cells to be destroyed.

CRediT authorship contribution statement

Yiwei Liu: Conceptualization, Investigation, Methodology, Data curation, Visualization, Formal analysis, Writing - original draft. **Xiang Li:** Conceptualization, Investigation, Formal analysis, Methodology, Writing - review & editing. **Zhao Tan:** Methodology, Formal analysis, Writing - review & editing. **Chunping Yang:** Conceptualization, Investigation, Methodology, Writing - review & editing, Supervision, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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