Triclosan degradation in sludge anaerobic fermentation and its impact on hydrogen production

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ABSTRACT

Triclosan (TCS), a widely used antibacterial agent, was accumulated at significant levels in waste activated sludge (WAS). To date, however, the interaction between TCS and sludge anaerobic fermentation was rarely reported. Hence, this work aimed to deeply understanding the degradation of TCS in sludge anaerobic fermentation and its impact on hydrogen production. Experimental results showed that ~45% of TCS was degraded in long-term anaerobic fermentation, with 2,4-dichlorophenol as its main intermediate. Based on the information from high performance liquid chromatography–mass spectrometry analysis, three pathways i.e., dichlorination, hydroxylation, and cleavage of ether bonds, were proposed for TCS degradation. It was found that the maximum hydrogen yield decreased from 18.6 to 12.8 mL/g VSS with the increase of TCS from 12 to 487 mg/kg TSS. One possible reason for the decreased hydrogen yield was that a part of hydrogen generated might serve as electron donors for TCS dichlorination. Besides, the presence of TCS significantly suppressed acidogenesis (an important step responsible for hydrogen generation). This inhibition to acidogenesis is likely due to that the high-affinity functional groups of TCS such as hydroxyl groups could bind to the active sites of acetate kinase (AK, a key enzyme in acidogenesis), which reduced the active sites available for original fermentation substrates. Microbial analysis revealed that TCS increased the relative abundances of potential contaminant decomposers such as *Guggeheimella* but inhibited the populations of hydrogen producers such as *Proteiniborus*, which was consistent with the results obtained by chemical analyses.

Keywords: Triclosan; Waste activated sludge; Anaerobic fermentation; Hydrogen production

1. Introduction

As a typical broad-spectrum antibacterial agent, TCS is extensively added to various personal care products and household products [1,2]. It was reported that annual consumption of TCS reached ~ 1,500 tons in the world [3,4]. Although European Union and USA interdicted the application of TCS in care products [5], other

regions such as China and India have not regulated TCS in these products yet. For example, the consumption of TCS is calculated to be ~1.3 mg/capita/day in China based on the analysis report [6]. TCS is still highly produced and consumed in these countries, which inevitably enters into environment. According to the literature, TCS is universally detected at ppt or ppb level in aquatic environments [7,8]. Its toxicity to aquatic organisms has attracted increasing attentions. For example, Ding et al.[9] found that 500 μg/L TCS caused severely inhibition (> 60%) in the growth of *Cymbella* sp. within 24 h. TCS exhibited teratogenic responses, hatching delay, and mortality in the larvae of zebrafish, with 0.42 mg/L of the 96 h median lethal concentration [10].

In fact, approximately 72-94% of TCS used would be collected by drain and then transferred to wastewater treatment plants (WWTPs), the main places for TCS removal to mitigate its aquatic environmental risks [11]. TCS entered into WWTPs is readily adsorbed onto microbes, and more than 50% of it would be transferred from wastewater to WAS in wastewater treatment processes, resulting in its significant accumulations in WAS [12–14]. It was documented that worldwide TCS content in digested sludge was typically in the range of 0.5 to 55 mg/kg [12,15,16]. Since the use of TCS has not been restricted in many countries (e.g., China and India), its content in WAS in these areas may further increase in the future. Thus, it is essential to evaluate the interaction between TCS and WAS treatment.

As a technology to effectively achieve WAS reduction and energy recovery (e.g., H_2 and short-chain fatty acids (SCFAs)), WAS anaerobic fermentation has recently attracted growing concerns [17–20]. WAS anaerobic fermentation involves a range of bioprocesses such as hydrolysis, acidogenesis, and acetogenesis driven by varieties of anaerobes (e.g., *Petrimonas* and *Clostridium*) [21–24]. As an antibacterial agent, TCS (> 30 mg/kg) might inhibit the activity or even cause the death of some key function microbes[25], and therefore it would be detrimental to anaerobic treatment processes, as observed by previous reports [26–28].

Several efforts were dedicated to revealing the fate of TCS in WAS (or soil) treatment processes or its potential impacts on the processes in the previous studies. The observation that TCS concentration in anaerobic soils did not decrease significantly within 70 days [29], indicating that TCS was non-biodegradable in anaerobic soils. Based on a 2-day mass balance on a real-life sewage plant, McAvoy et al. [30] also verified that TCS was not degraded in anaerobic sewage sludge treatment. However, Samaras et al. [31] reported that more than 45% TCS loss was observed in two lab-scale anaerobic digesters after 20 days under thermophilic and mesophilic anaerobic conditions, suggesting that TCS was biodegradable in WAS anaerobic digestion. Tohidi and Cai [32] demonstrated that 18.6%-23.0% of TCS mass loss was detected in a WAS anaerobic digester in 19 days, indicating its biodegradability as well. Recently, Wang et al. [5] reported that ~37% TCS was biodegraded in WAS anaerobic digestion after ~40 days, and the presence of TCS deteriorated methane yield. Methane yield decreased from 108.4 to 95.2 mL/g VSS as TCS content increased from 200 to 550 mg/kg TSS.

These significant investigations mainly focused on either the evaluation of TCS's removal efficiency in anaerobic treatment or its negative effect on anaerobes (i.e., methanogens) in sludge anaerobic digestion. To date, however, the fate of TCS during sludge anaerobic fermentation and the mechanism of how TCS affects WAS anaerobic fermentation have not yet been comprehensively investigated. Besides, the conclusions obtained in these references are controversial, resulting in several key questions to be clarified systematically. These questions primarily include: Is TCS really degraded in anaerobic fermentation processes? If it is, which substances are the main degradation intermediates and which metabolic pathways are involved in the degradation? Do TCS and its main metabolic intermediates affect the bioprocesses in anaerobic fermentation? If so, how does TCS impact the predominant microbial community during sludge anaerobic fermentation?

Through addressing the issues above, the objective of the present study is to fully investigate the interaction between TCS and sludge anaerobic fermentation. On one hand, the degradation and transformation of TCS in

anaerobic sludge system were assessed, aiming to understand the fate of TCS during anaerobic treatment deeply. On the other hand, the details of whether and how TCS affects sludge anaerobic fermentation were investigated. The implications of understanding the impact of TCS in sludge anaerobic fermentation would provide theoretical basis and reference for the future manipulation of TCS-rich sludge.

2. Materials and methods

2.1 Sources of WAS and TCS

The WAS (a collection of 50 L samples) used in this experiment was taken from Kaifu Sewage Treatment Plant in Changsha, China. The obtained WAS was first passed through a 2 × 2 mm sieve to remove sand, wood chips and other impurities, and then settled for 1 d. After the supernatant being poured out, the settled WAS was stored in a 4 °C refrigerator for subsequent experiments. The settled WAS used in this study owned the following major properties: pH 6.8 ± 0.2, volatile suspended solids (VSS) 18050 ± 180 mg/L, total suspended solids (TSS) 30080 ± 330 mg/L, soluble chemical oxygen demand (SCOD) 400 ± 14 mg/L, soluble carbohydrate 23 ± 1 mg COD/L, soluble protein 261 ± 9 mg COD/L, C/N 7:1, and TCS content 12 ± 2 mg/kg TSS. Extra TCS (purity over 97%) was provided by Shanghai Rhawn Chemical Industry Park, China.

2.2. Batch experiment for anaerobic fermentation of WAS at different TCS levels

The batch experiment was performed in 5 replicate reactors (500 mL each). 300 mL of the above settled WAS were first added to each reactor. Different extra TCS were then placed into the reactors to obtain initial TCS content of 12 ± 2, 97 ± 5, 182 ± 9, 322 ± 17 or 487 ± 25 mg/kg TSS. Notably, the first reactor had no additional TCS, with the background value of 12 ± 2 mg/kg TSS. Among them, 12 ± 2 and 97 ± 5 mg/kg TSS of TCS were selected as environmentally relevant levels. Considering the increasing usage of TCS in many developing regions (e.g., China and India), three high TCS levels (i.e., 182 ± 9, 322 ± 17, and 487 ± 25 mg/kg TSS) were also selected to obtain more comprehensive information. Afterwards, all the reactors were injected

with nitrogen gas for 5 minutes, then covered and sealed with rubber. Finally, the reactors were positioned in an air-bath shaker (150 rpm) at 35 ± 2 ℃ for 21 d. Since alkaline fermentation (especially pH 10) was reported to be an effective approach for inhibiting hydrogen consumption and obtaining high hydrogen production [33,34], pH in each reactor was controlled at 10 via a programmed logic controller.

2.3. Long-term semi-continuous test for WAS anaerobic fermentation at different TCS levels

For deeply understanding the interaction between TCS and WAS anaerobic fermentation from microbial analysis, two reactors of long-term semi-continuous with two representative TCS dosages were also conducted. One as the control group, corresponded to reactor of WAS without extra TCS addition (i.e., initial WAS TCS content of 12 ± 2 mg/kg TSS), and the other was an experimental reactor which was fed with WAS with initial WAS TCS content of 322 ± 17 mg/kg TSS. In this test, 12 mg/kg TCS was considered as the background value. Due to budget limitation, however, only 322 mg/kg TCS was selected, because obvious impact on microbial community were expected at such high TCS levels. In future, more long-term semi-continuous fermenters with different TCS additions should be operated to understand the impact of TCS on microbial community comprehensively.

The working volume of the two semi-continuous reactors was 1000 mL each. Each reactor contained 600 mL WAS with or without extra TCS addition, and all other fermentation conditions followed the above. From the results of the batch experiment in Section 2.2, 12 days was determined as the sludge retention time for the two long-term reactors. Therefore, 50 mL fermentation mixture was daily extracted from each reactor and replaced with new WAS (containing the equivalent of TCS) of the same volume. Nitrogen gas was then sparged into the two reactors for 5 min, and the two reactors were re-capped and re-sealed before they were put back into the air-bath shaker. During the entire experiment, hydrogen yield was measured and recorded daily. After 65 d operation, hydrogen yield from the two reactors reached relatively stable. Thus, microbial analyses

were performed. In addition, the third reactor fed with 300 mL sludge and 322 ± 17 mg/kg TSS TCS was operated to determine the long-term degradation efficiency of TCS. The experiment conditions were the same as those depicted in Section 2.2 except that the fermenter was run for 65 d (based on the above result). Sludge TCS content was also detected regularly.

2.4. The impacts of TCS and 2,4-dichlorophenol (2,4-DCP) on each process related to hydrogen production during sludge anaerobic fermentation

This batch test was used to investigate the impacts of TCS and 2,4-DCP on each fermentation process relevant to hydrogen production. The two groups used for the test were Group-A and Group-B. pH in Group-A was not controlled while pH in Group-B was controlled at 10.0. By comparing the changes in SCOD, soluble proteins, and soluble polysaccharides with presence or absence of TCS and 2,4-DCP, there was an assessment in real WAS for the effect of TCS and 2,4-DCP on WAS solubilization. The specific degradation rates of model substrates in the presence or absence of TCS and 2, 4-DCP were compared to reveal the impact of TCS and 2, 4-DCP on other biological processes (including hydrolysis, acidogenesis, and acetogenesis). The experimental procedures were detailed in Supporting Information (SI).

2.5. Model-based analysis

In this work, the correlation between the cumulative hydrogen production (H, mL/g VSS) and fermentation time (t, d) was described by Gompertz model function (Eq. (1)) according to the references [35–37]. Origin 9.0 software was employed in calculating several kinetic parameters (i.e., Hm, maximum hydrogen yield potential, mL/g VSS; λ, lag-phase time of hydrogen production; and Rm, Hydrogen production rate, mL/(gVSS•d)).

$$H = Hm \cdot exp\{-exp[Rm \cdot e \cdot (\lambda - t)/Hm + 1]\} \quad (1)$$

The inhibition constant calculated by Eqs. (2) and (3) can reflect the TCS effect on the degradations of

dextran, glucose, and butyrate.

$$C_0 - C_t = C_0 \cdot [1 - exp(K \cdot t)] \quad (2)$$

Where C_0 and C_t are the initial concentration of model substrates and the concentration of model substrates at t day fermentation of model substrates, respectively, t is the fermentation time (d), and K represent the degradation kinetic rate of model substrates (mg/g VSS·d).

$$K_{s,i}/K_{s,0} = 1/(1 + Ii/X_{s,i}) \quad (3)$$

Where K is the removal efficiency as show in Eq. (2). In addition, subindex "s" refers to the model substrate, and "i" is the inhibitor. Ii and Xs,i are the inhibitor concentration (mg/g VSS) and the related inhibition constant (mg/g VSS), respectively.

2.6. Analytical methods

2.6.1. Conventional parameters

The standard methods were used for analysis of COD, TSS, and VSS [38]. Polysaccharides and proteins were measured by the phenol-sulfuric method with glucose as the standard and the Lowry-Folin method with Bovine Serum Albumin as the standard, respectively [39]. 0.2 mL of the samples was injected into a gas chromatograph with gastight syringe to detect hydrogen and methane fractions in gas samples, and our previous publication [40,41] have exhibited the relevant collection and measurement procedures detailedly. Gas chromatograph with flame ionization detector (HP5890, GC) with 1.0 μL injection volume was applied to ascertain SCFAs of fermentation samples, and the measurement method was described in the literature [42]. The alterations of fermentation liquid were determined by excitation emission matrix (EEM) fluorescence spectroscopy (F-4600 FL spectrophotometer, Hitachi, Japan) according to the literature [43–45]. The activity of key enzyme (i.e., acetate kinase) was measured using the method described in the literature [39].

2.6.2. Measurement of TCS and its degradation products

The concentration of TCS and 2,4-DCP were quantified by high performance liquid chromatography (HPLC) system (Agilent 1200) equipped with a C18 column with UV detection. The sludge samples, which should be pretreated before being injected into HPLC system, were defined as pretreated samples. Firstly, 10 mL of the fermentation samples were taken out from the reactor fed TCS at different fermentation time, centrifuged, collected the supernatant and the sediment. The collected sediment dried under vacuum, and then weighted 0.10 g dissolved in 10 mL methanol, vibrated for 5 min, and followed by ultrasonic for 0.5 h. After re-centrifugation at 8000 rpm and 4 ℃ for 10 min, the supernatant of the sample was gathered in a centrifuge tube. The above steps are performed twice repeatedly to treat the residue. At last, the supernatant was filtered with organic filter (0.22 μm) for removal of impurities for subsequent measurement. The mobile phase of TCS was methanol (77%) and purified water (23%), with an injection volume of 10 μL at a flow rate of 1.0 mL/min and a detection wavelength of 281 nm [46]. The mobile phase of 2,4-DCP consisted of purified water (15%) and methanol (85%) at flow rate of 1.0 mL/min, and the detection wavelength was 290 nm [47]. According to method guidelines from the literature [48], the limits of detection and quantification were determined as 2.6 and 8.7 μg/L for TCS, and 1.4 and 4.6 μg/L for 2,4-DCP, respectively. Standard addition experiments of TCS or 2,4-DCP were performed by sludge samples with each sample being determined in triplicate [49,50]. The samples were collected and analyzed using the same methods as described above. Average recoveries (n = 3) and relative standard deviation were 91-107% and 6-11% for TCS, and 90-102% and 2-5% for 2,4-DCP, respectively.

In addition, identification the major intermediates of TCS was performed via high performance liquid chromatography–mass spectrometry (HPLC-MS) method [51]. The detailed procedure was described in SI.

2.6.3. Microbial community

The diversity of the microbial community in the two long-term semi-continuous reactors was assessed by Illumina Miseq sequencing. The fermentation samples were extracted from two reactors and then centrifuged at 10000 rpm for 5 min. Depending on the operating manual, the total genomic DNA was extracted from the samples using the Fast DNA Kit (MoBio Laboratories). Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was employed to analyze the quantity and purity of DNA. The primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used to target the V3-V4 regions of bacterial 16Sr DNA genes. The DNA samples were sequenced by Illumina miseq sequencing (Illumina Miseq PF300 platform).

2.7. Statistical analysis

All the batch experiments were conducted in triplicate. All figures in this work were constructed using Origin 9.0. The significance of the results was evaluated by the analysis of variance, and $p < 0.05$ was regarded as statistically significant.

3. Results and discussion

3.1. Alteration of total TCS content in WAS anaerobic fermentation

Fig. S1A displays HPLC chromatograms of pretreated samples extracted from the batch test fermenter fed with 322 ± 17 mg/kg TSS TCS at various fermentation time. Based on the standard chromatogram, the second distinct adsorption peak (appears at ~ 6.2 min in the chromatogram) was identified as TCS (Fig. S2A). This absorption peak gradually decreased as anaerobic fermentation progressed, suggesting that TCS was biodegraded by some anaerobes in sludge anaerobic fermentation. Quantitative counting further showed that with an increase of fermentation time, total TCS content (including liquid phase and sludge phase) reduced significantly. In the 322 ± 17 mg/kg TSS TCS reactor (initial TCS was 2.91 ± 0.15 mg), for example, 2.55 ± 0.12 mg TCS was measured on 7 d fermentation. After 21 d fermentation, 2.04 ± 0.07 mg TCS was detected,

indicating that 29.8% of TCS was biodegraded in the batch fermentation process (Fig. 1A). Similar observations were also made in other batch tests, with 27.3-35.4% of TCS was degraded (Table S1). The biodegradation of TCS could be further confirmed by long-term test (Fig. 1B). In the experimental reactor, total TCS content decreased from 2.91 ± 0.15 to 1.87 ± 0.06 mg with the fermentation time increasing from 0 to 30 d. After 65 d operation, 1.60 ± 0.05 mg TCS was measured. The result suggests that ~45.0% of TCS was degraded in the long-term test.

There are some opposite conclusions documented in the literatures in terms of TCS biodegradation during anaerobic treatment. McAvoy et al. [30] found that no variation was measured in TCS concentration in anaerobic digestion process. However, Tohidi and Cai [32] demonstrated that ~ 23% of TCS was lost in 19-d anaerobic digestion, while Zhou et al. [52] reported that the elimination for TCS was above 70% during sewage sludge anaerobic digestion process by using ultrasonic pre-treatment. The results obtained in our work supported the latter but not the former, clearly exhibiting that TCS can be partially biodegraded in anaerobic treatment. There are several possible reasons for these inconsistent findings. The concentration of TCS used in the study of McAvoy et al. [30] was merely ~0.53 mg/kg TSS, and such level of TCS might be too low to be detected. Another probable explanation is that the use of sludge spiked or unspiked with micropollutants might lead to different degradation efficiencies, as observed by Gonzalez-Gil et al. [53]. Additionally, sludge with pre-treatment (e.g., ultrasonic pre-treatment) might also cause higher removal efficiency of TCS than that without pre-treatment during sludge anaerobic treatment.

3.2.The major intermediates and possible degradation pathways of TCS

HPLC-MS was used to identify the major intermediates of TCS degradation. Fig. 2A exhibits HPLC/MS scan spectra of the pretreated samples (on 5 d and 21 d fermentation) taken from the batch reactor fed with 322 ± 17 mg/kg TSS TCS WAS. It can be seen that seven peaks (namely, P0-P6) were found in the spectra, with

their retention time being 6.56 min, 5.95 min, 5.73 min, 5.16 min, 1.88 min, 0.96 min, and 0.94 min, respectively. This indicates that the samples mainly contain seven compounds, which were consistent with the comparison results obtained in the control reactor (i.e., without additional TCS) with the reactor fed with 322 ± 17 mg/kg TSS TCS (Fig. S1B). Their major $[M-H]^-$ product ions were respectively m/z 287, m/z 269, m/z 219, m/z 253, m/z 161, m/z 143, and m/z 127. According to the literature, these compounds were identified as TCS, 5-chloro-2-(2-hydroxy-4-chorophenoxy) phenol, 2-(4-chlorophenoxy) phenol, 2-(2,4-dichlorophenoxy) phenol, 2,4-DCP, 4-chlorocatechol, and 4-Chlorophenol, respectively. As anaerobic fermentation progressed, the relative intensity of P0 decreased, accompanied by increases of other peaks. This suggests that TCS was degraded in fermentation process (as shown in Fig.1), with six intermediates or products being majorly produced. It can also be observed that P4 (i.e., 2,4-DCP) was the major intermediate of TCS degradation, which was also detected usually by other investigators in TCS degradation experiments [54,55]. Under stable operatopn, 13 ± 2 mg/kg TSS of 2,4-DCP was measured in the long-term semi-continuous fermenter (adding 322 ± 17 mg/kg TSS TCS) (quantitative information see Fig. S2 for details).

To further identify the possible structure of 2,4-DCP, mass spectrometric (MS)/MS analysis was employed. This method was widely examined by other researchers [56–58] and also by us. For example, an ion cluster with m/z 287/289/291/293 was detected at 6.56 min, with a chlorine isotope abundance ratio of 27: 27: 9: 1 being calculated, suggesting the presence of three Cl atoms in this compound. The fragment ions at m/z 251,161 and 125 correspond to the loss of HCl, C_6H_2O, and HCl, respectively (Fig. S3). The proposed structure of TCS based on MS/MS analysis was the same as that of standard TCS provided bythe company. From Fig. 2B, it can be found that the chromatographic peak with a retention time of 1.88 min contains an ion cluster at m/z 161/163/165, indicating that 2,4-DCP contains two Cl atoms. The presence of a fragment ion peak at m/z 125 indicates that the parent ion (161) lost HCl. Based on the analyses above, the structure of 2,4-DCP

was therefore proposed (Fig. 2B). According to the similar analysis, the structures of other intermediates were also obtained, with the details being shown in Fig. S3.

On the basis of these intermediates detected, three pathways were proposed herein for TCS degradation in WAS anaerobic fermentation. These pathways mainly contained dichlorination (Pathway A), hydroxylation (Pathway B), and cleavage of ether bonds (Pathway C), with details being shown in Fig. 3.

In Pathway A, TCS was first converted into 2-(2,4-dichlorophenoxy) phenol (P3, m/z 253) through reductive dichlorination, which was mainly caused by nucleophilic attack of electrons on carbon cations [59]. 2-(2,4-dichlorophenoxy) phenol (P3, m/z 253) was transformed to 2-(4-chlorophenoxy) phenol (P2, m/z 219) and 2,4-DCP (P4, m/z 161) via dichlorination and homolytic reaction of the C-O bond, respectively. The generated 2-(4-chlorophenoxy) phenol (P2, m/z 219) could be further transformed to 4-Chlorophenol (P6, m/z 127) via ether linkage cleaving. In Pathway B, 5-chloro-2-(2-hydroxy-4-chorophenoxy) phenol (P1, m/z 269) was produced through hydroxylation. Apart from dichlorination (Pathway A) and hydroxylation (Pathway B), TCS could also be degraded through cleavage of ether bonds [48], with two intermediates, i.e., 2,4-DCP (P4, m/z 161) and 4-chlorocatechol (P5, m/z 143), being produced, respectively (Pathway C). It should be noted that 2,4-DCP was produced in both Pathway A and Pathway C, which might be the reason why it was the major intermediate of TCS's biodegradation in such systems. Some scientists pointed out that transformation product formation was affected by the initial concentration of micropollutants [60], thus the major intermediates and possible degradation pathways of TCS proposed in Fig. 3 necessitated to be further confirmed by other initial TCS levels in the future. Besides, in the future, more efforts should be operated to comprehensively reveal the respective contributions of each possible pathway to the TCS degradation.

3.3. Hydrogen production from WAS anaerobic fermentation at different TCS contents

Fig. 4A shows the cumulative curve of hydrogen production from sludge anaerobic fermentation at different TCS levels under alkaline condition (pH 10). It was found that the cumulative curves of hydrogen production from the samples showed a similar trend. In the first 12 days, hydrogen yield in the fermenters gradually increased, and hydrogen production did not increase significantly ($p > 0.05$, see Table S2) after 12-day fermentation. This suggests that the optimum fermentation time was 12 days in all the scenarios. With TCS contents enhanced from 12 ± 2 to 487 ± 25 mg/kg TSS, the maximum hydrogen yield decreased significantly from 18.6 ± 0.4 to 12.8 ± 0.3mL/g VSS ($p < 0.05$, see Table S3). Besides, COD mass balance of the fermentation reactors with different TCS addition in batch tests were placed in Table S5.

To deeply analysis the influence of TCS on hydrogen production, the experimental results was described via Gompertz empirical model. The Gompertz model fitted the experimental data well (Fig. 4A), with R^2 being > 0.98 in all the cases. Based on the model, the pivotal kinetic parameters, the maximum hydrogen production potential (Hm), hydrogen yield rate (Rm), and lag-phase time of hydrogen yield (λ) were calculated. Although lag-phase time of hydrogen yield (λ) did not vary obviously, the maximum hydrogen production potential (Hm) and hydrogen yield rate (Rm) decreased respectively from 17.8 mL/g VSS and 5.1 mL/g VSS·d to 12.2 mL/g VSS and 3.5 mL/g VSS·d, with the increase of TCS from 12 ± 2 to 487 ± 25 mg/kg TSS (Table 1). It also showed that the maximum hydrogen yield potential decreased exponentially with the increase of TCS (Fig. 4B). All the experimental and model results demonstrated that TCS inhibited the hydrogen production from sludge dark fermentation.

3.4. How does the presence of TCS inhibit hydrogen yield?

Hydrogen yield was closely related to several steps of WAS anaerobic fermentation, including sludge solubilization, hydrolysis, acidogenesis, and acetogenesis [61]. Substrates can be supplied to hydrogen producers through the dissolution and hydrolysis of sludge, and acidogenesis and acetogenesis produce hydrogen directly.

Therefore, the potential impact of TCS on these steps was assessed. Since 2,4-DCP was demonstrated to be the major intermediate of TCS degradation, its impact on these steps was also evaluated. In the literature, the efficiency of sludge solubilization is usually evaluated by determining the concentrations of soluble COD, carbohydrates and proteins in real sludge fermentation liquid while the rates of other biological steps are usually measured by batch tests using model substrates such as dextran, glucose, and butyrate due to their dynamic in real sludge fermentation systems [62].

Previous investigation found that low TCS levels (< 500 mg/kg TSS) had no obvious effect on sludge solubilization [46], and this was also demonstrated by the data obtained in our experiment (Fig. S4). It should be noted that relatively high TCS levels (> 1000 mg/kg TSS) could inhibit sludge solubilization [5], which was possibly due to high toxicity of TCS at this concentration. The results from batch tests using model substrates showed that with an increase of TCS from 12 ± 2 to 487 ± 25 mg/kg TSS, the specific degradation/consumption rates of dextran and butyrate in alkaline (pH 10) fermentation slightly decreased from 239.2 ± 9.6 and 192.6 ± 6.8 to 228.3 ± 8.5 and 175.0 ± 6.5 mg/g VSS·d (Fig. 5A-I). However, further calculations exhibited these variations were statistically insignificant ($p > 0.05$). Similar observations were also made in anaerobic fermentation without pH control (Fig. 5A-II). The results suggest that the presence of TCS did not affect hydrolysis and acetogenesis steps significantly, as observed by Wang et al. [5].

It was reported previously that the specific degradation rate of glucose was significantly affected by high TCS [5]. This work also confirmed the conclusion. When TCS increased from 12 ± 2 to 487 ± 25 mg/kg TSS, the specific degradation rate of glucose in anaerobic fermentation with pH 10 and pH uncontrol decreased from 203.3 ± 7.5 and 269.0 ± 9 to 174.2 ± 6.3 and 233.2 ± 7.9 mg/g VSS·d, respectively (Fig. 5A). This indicates that the significant inhibition to acidogenesis (an important step in hydrogen generation) by the presence of TCS was the main reason for TCS reducing hydrogen production, which was consistent with the result

obtained in Fig. 4.

Hydrogen production from acidogenesis is directly associated with several enzymatic activities. As a key enzyme in acidogenesis, AK plays a critical role in the conversion from pyruvate to SCFAs and hydrogen [63,64]. The two monomers of AK dimer are shown in green and blue, respectively (Fig. 6), with the monomers being formed by the C-terminal and the N-terminal domains [65]. The active sites of AK, which bind with substrates, are located between N and C domains (cavities located in the stereoviews of active sites of Fig. 6). It was observed that the combination of AK and substrates depends on the affinity of substrate functional groups and the molecular size of substrates [66,67]. When TCS is present in WAS anaerobic fermentation systems, TCS could bind to the active sites of AK due to its small molecular size and high-affinity functional groups (i.e., hydroxyl groups) [68]. It was also demonstrated through the analysis of microbial key enzyme (see Table S4 for details). As a result, the active sites available for original fermentation substrates in TCS added systems would decrease, as compared with the system without TCS addition. This may be the reason why the presence of TCS suppressed acidogenesis. Besides, Wang et al. [69] found that TCC facilitated the release of substances and acidification but decreased methanogenesis process. The possible reason for this is that TCS has the different functional groups (i.e., hydroxyl groups) with TCC. An alternative reason is that the different experimental methods of TCS and TCC.

It can be seen that 2,4-DCP at the tested level did not affect all the biological steps significantly (Fig. 5B). This is likely due to the low level (~13 mg/kg TSS) used in the tests. Since the half maximal effective concentration value of TCS and 2,4-DCP to activated sludge microorganisms was respectively ~20 and ~74 mg/L [70,71], an alternative explanation is that fermentation anaerobes are less sensitive to 2,4-DCP, as compared with TCS.

Besides, the presence of TCS might promote hydrogen consumption. It is generally thought that reductive dichlorination requires chlorophenols as electron acceptors with either simple organics (e.g., SCFAs) or H_2 as electron donors [72,73]. This indicates that a part of hydrogen generated may serve as electron donors for TCS dichlorination in WAS anaerobic fermentation system. This might be another reason for TCS reducing hydrogen yield. However, it is hard to differentiate the respective contributions of SCFAs and hydrogen to reductive dichlorination, because they are concurrently produced and co-existed in the system (as shown in Table S5). Thus, further efforts are required to be performed in the future.

3.5.TCS affect the microorganisms in the long-term semi-continuous fermenters

To figure out TCS's impact on anaerobic microorganisms, microbial diversity in the long-term semi-continuous fermenters was finally analyzed using Illumina sequencing. These two reactors were fed with WAS with different TCS content (i.e., 12 ± 2 mg/kg TSS in the control and 322 ± 17 mg/kg TSS in the experimental fermenter). It was measured that the number of operational taxonomic units (OTUs) was 365 in the control and 355 in the experimental reactor, with 339 being shared by the two reactors (Fig. S5). Based on the analysis of Alpha diversity and Simpson index, the increase of TCS inhibited the microbial diversity but did not significantly change the microbial structure (Fig. S6). Wang et al. [5] detected, however, that total OTUs of the control were less than that of the experimental reactor (i.e., 1200 mg/kg TCS) (881 versus 1175), which was inconsistent with the data obtained in this work. It should be emphasized that most of the increased microorganisms (e.g., *Sporosarcina*) belonged to the potential TCS decomposers. This indicates that the increase of TCS enhanced TCS degraders but reduced other microbes.

The two microbial communities were mainly composed of four phyla, i.e., Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, accounting for more than 85% in both the two fermentation reactors (Fig. S7). It was reported that several microorganisms affiliated to Bacteroidetes were hydrolytic microbes [74], while a

part of microbes responsible for production of SCFAs and hydrogen belonged to Firmicutes and Actinobacteria [75–77]. For instance, the relative abundances of microbes belonged to Firmicutes decreased from 39.35% in the blank to 35.46% in the experimental fermenter, reflecting that Firmicutes were inhibited by the presence of TCS. Similar results were also found in the study by Wang et al. [5], in which they observed that the relative abundances of Firmicutes in the experimental reactor decreased by 35.62% in comparison to the control. Since the lower TCS (322 ± 17 mg/kg TSS) was selected in this study, it could be supposed that the toxicity to Firmicutes increased as TCS increased.

Fig. 7 further exhibits the distributions of bacterial abundances at the genus level in the two reactors. Twelve genera of microorganisms, which were accountable for the production of SCFAs and hydrogen, were observed in the two reactors. For instance, as a typical hydrogen-SCFAs producer [78], the abundance of *Proteiniborus* was 12.09% in the control whereas the corresponding population was 9.64% in the experimental fermenter. The abundance of *Corynebacterium*, which was reported to be SCFAs producers [79,80], decreased from 12.55% in the control to 3.80% in the experimental fermenter. Further calculations revealed that total abundance of these twelve genera was 44.00% in the control and 34.90% in the experimental fermenter. In the experimental reactor (i.e., the reactor fed with 322 ± 17 mg/kg TCS), TCS could bind to the active sites of AK (a key enzyme in acidogenesis), thus these sites available for original fermentation substrates will decrease (see Fig. 6 for details). This indicates that TCS's suppressions to acidogenesis were much more severe than that of other bioprocesses (e.g., hydrolysis and acetogenesis), which could be further supported by the degradation rates in model experiments (Fig. 5). As a result, the presence of TCS would reduce the amounts of SCFAs & hydrogen producers, which was consistent with the previously reported observations [5].

Apart from hydrogen-SCFAs producers, other functional microbes were measured at significant levels as well. It can be sedeen that *Guggeheimella*, which could biodegrade different complex organic pollutants (e.g.,

petroleum hydrocarbons) under anaerobic conditions [74,78], increased from 7.41% in the control to 11.88% in the experimental reactor (Fig. 7). In addition, the populations of several contaminant decomposers such as *Conexibacter*, *IMCC26207*, and *Soehngenia* [81–83], in the control was also lower than those in the experimental reactor, which supported TCS biodegradation data shown in Fig.1 and Fig.2. The total abundance of potential contaminant degradation microorganisms (e.g., *Guggeheimella and Conexibacter*, etc.) increased from 20.06% in the control to 29.38% in the experimental reactor, indicating that these microorganisms might be TCS biodegradation microorganisms in anaerobic fermentation systems. In the reference, Wang et al. [5] reported that the relative abundances of *Sporosarcina*, which was reported to have ability to degrade some contaminants such as phenanthrene and trichloroethylene[84], increased from 0.001% in the control to 44.73% in the experimental reactor. The results implied that *Sporosarcina* might be potential microbes for the degradation of TCS. It is worth noting that such obvious increases in TCS degraders might be the main reason for high TCS (i.e., 1200 mg/kg TSS) seriously changing the richness and diversity of species. Considering that the TCS concentration in this work was significantly lower than that used in the previous study (322 versus 1200 mg/kg TSS), such strong inhibitions or promotions to microorganisms were not detected in the present research. Since pH 10 and pH 7 were operated in the current and previous work, an alternative reason for the inconsistent changes in microbial community was that alkaline treatment (i.e., pH 10) severely inhibited the activity of many microorganisms already [77,85].

3.6.Implications for sludge treatments

As a typical broad-spectrum antibacterial agent being extensively used, TCS is substantially released in the environment. Thus, the fate and impact of TCS in biological processes have attracted growing attention. Although several efforts were dedicated to assessing whether TCS could be degraded in anaerobic treatment, the results were inconsistent. This study represented comprehensive understanding of the interaction between

TCS and WAS anaerobic fermentation, which contributed to addressing the controversy in terms of TCS degradation in anaerobic treatment.

It was found that although part of TCS could be degraded, ~55% of TCS was still detected at the end of WAS anaerobic fermentation (Fig. 1). The remaining TCS inevitably enters into the environment, which brings potentially environmental risks. To mitigate the toxicity of TCS to WAS anaerobic fermentation and subsequent natural environment, strategies such as advanced oxidation-based pretreatment that could effectively enhance the degradation of TCS in the fermentation process are urgently required. It was reported that advanced oxidation-based pretreatment, e.g., peroxymonosulfate and calcium peroxide pretreatment not only decreased the species and total detection frequency of recalcitrant organics but also enhanced the disintegration of sludge cells effectively, which benefited WAS anaerobic fermentation or digestion substantially [45,86]. The feasibility of such advanced oxidation-based pretreatment to promote TCS degradation necessitates to be assessed in the future.

4. Conclusions

TCS could be partially biodegraded in WAS anaerobic fermentation through three possible pathways i.e., dichlorination, hydroxylation, and cleavage of ether bonds, based on the quantitative calculation of total TCS content (including the liquid and sludge phases) variations and HPLC–MS analysis. The toxicity of TCS to anaerobes was higher than its major metabolic intermediate (i.e., 2,4-DCP). Anaerobic fermentation could reduce the toxicity of TCS, but the remaining TCS accumulated in WAS would bring potential environmental risks. Therefore, it will be necessary to combine the pre-treatment methods to improve its removal efficiency in the future. This study demonstrated that high TCS (> 182 mg/kg TSS) in sludge fermentation systems inhibited hydrogen yield significantly through suppressing acidogenesis at the first time. TCS's inhibition may be related to the binding of the key enzyme active sites (i.e., AK, a key enzyme in acidogenesis) and thus the

active sites available for original fermentation substrates decreased, which resulted in inhibition to acidogenesis. The presence of TCS decreased the microbial diversity, decreased the populations of hydrogen producers, but enhanced the abundances of complex organic degradation microbes.

Acknowledgments

This study was financially supported by the project of National Natural Science Foundation of China (51779089) and the Science and Technology Innovation Project of Hunan Province (2018SK2028).

Appendix A. Supplementary data

This file contains analytical methods Text S1-S2, Figure S1-S7, and Table S1-S5.

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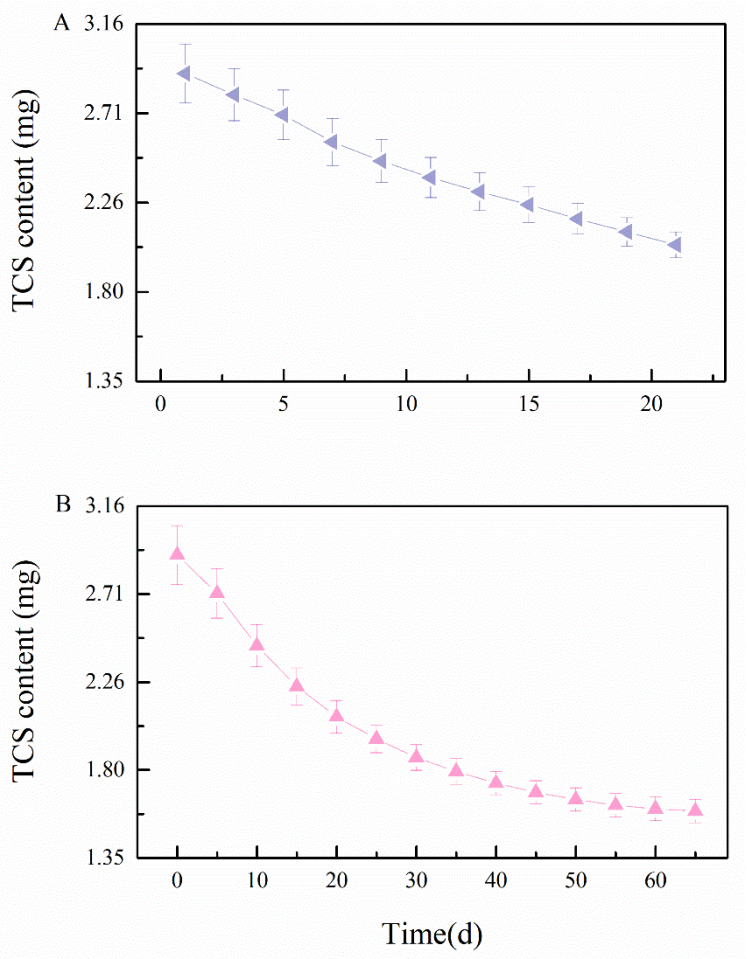
Table 1. Estimated Hm, Rm, λ and R^2 at different TCS levels using Gompertz equation.[a]

TCS level (mg/kg TSS)	Hm (mL/g VSS)	Rm (mL/(g VSS • d))	λ (d)	R^2
12 ± 2	17.8 ± 0.3	5.1 ± 0.1	0.37 ± 0.02	0.9846
97 ± 5	16.0 ± 0.3	4.5 ± 0.1	0.35 ± 0.02	0.9845
182 ± 9	14.8 ± 0.3	4.2 ± 0.1	0.34 ± 0.01	0.9849
322 ± 17	13.3 ± 0.2	3.8 ± 0.1	0.32 ± 0.01	0.9849
487 ± 25	12.2 ± 0.2	3.5 ± 0.1	0.31 ± 0.01	0.9846

[a] The results are average of triplicate tests.

1



1

2 **Fig. 1. The variation of total TCS content (including liquid phase and sludge phase) in batch (A) and**
3 **long-term (B) tests. Samples were collected in the batch reactor or the long-term reactor fed with 322**
4 **± 17 mg/kg TSS TCS (i.e., the experimental reactor). Error bars represent standard deviations of trip-**
5 **licate test.**

6

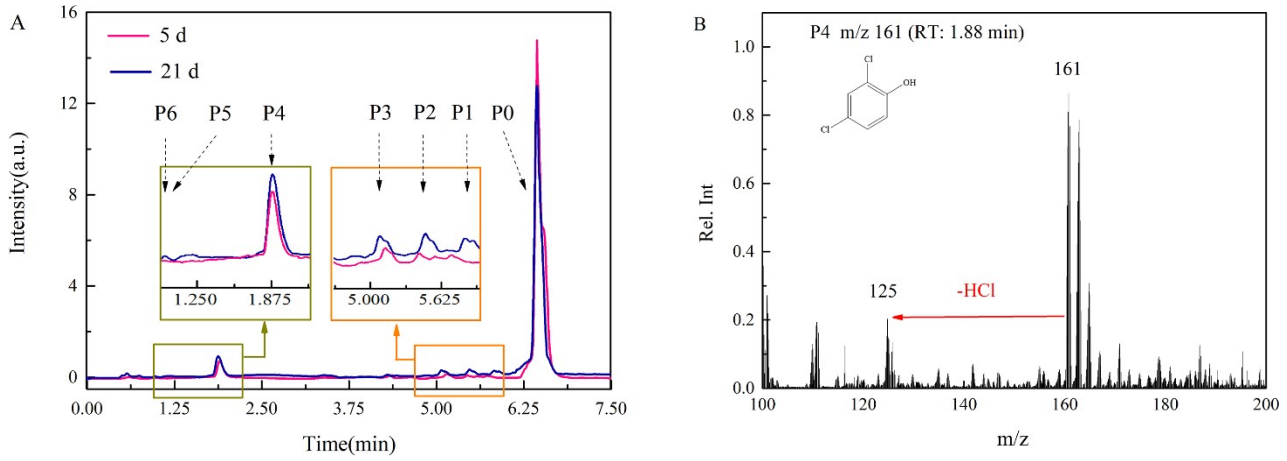


Fig. 2. HPLC/MS scan spectra of the pretreated samples taken from the batch reactor fed with 322 ± 17 mg/kg TSS TCS WAS (A), and product ion spectrum of 2,4-DCP (B). Samples were obtained on 5 d and 21 d fermentation, respectively.

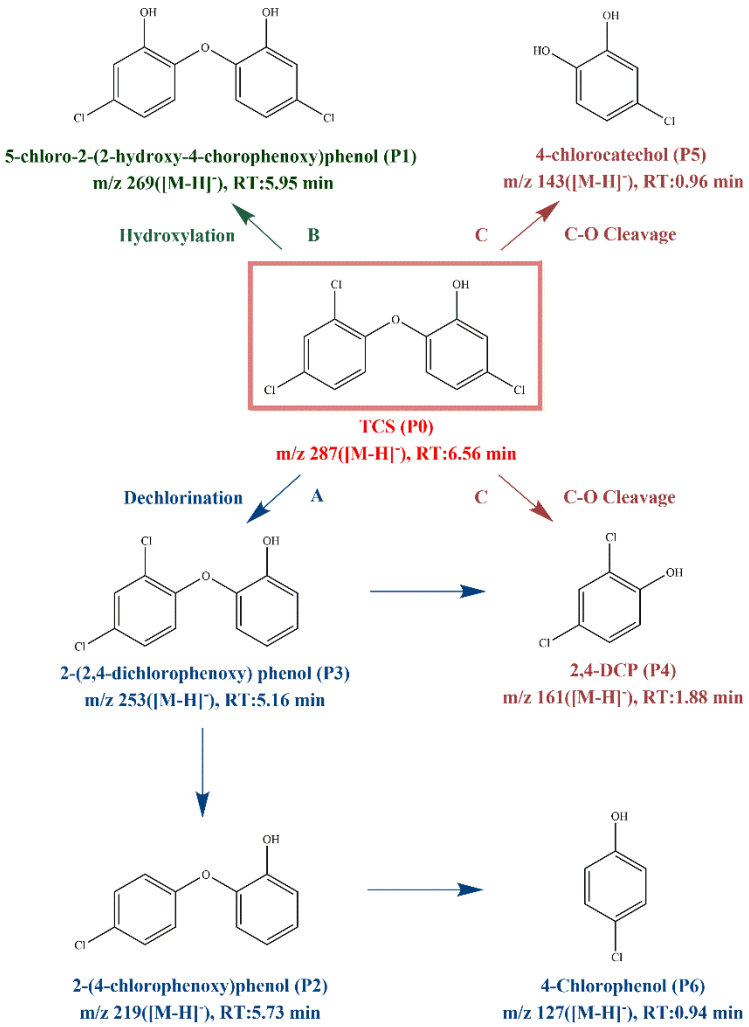
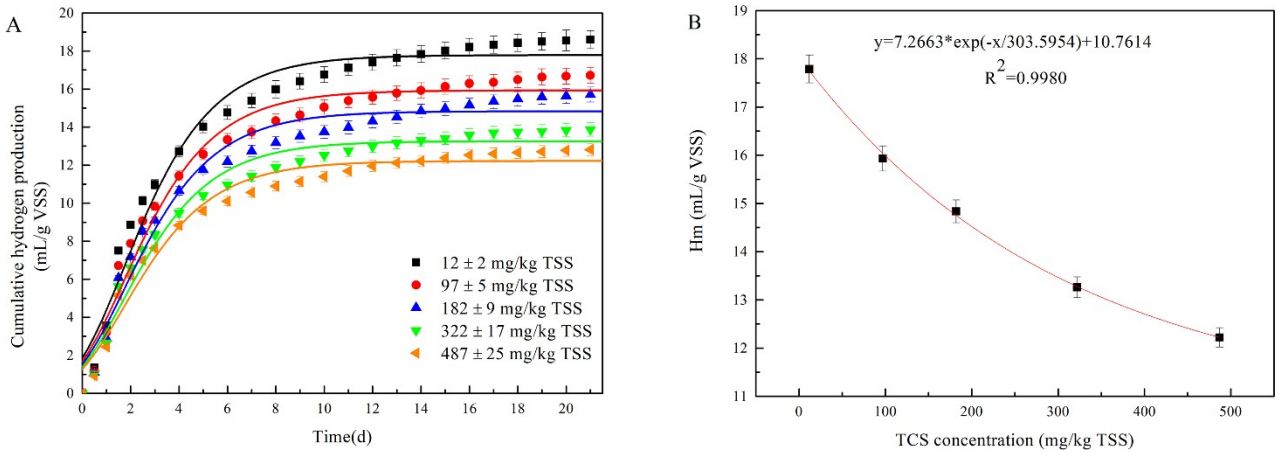


Fig. 3. The proposed pathways of TCS degradation in WAS anaerobic fermentation systems.



15

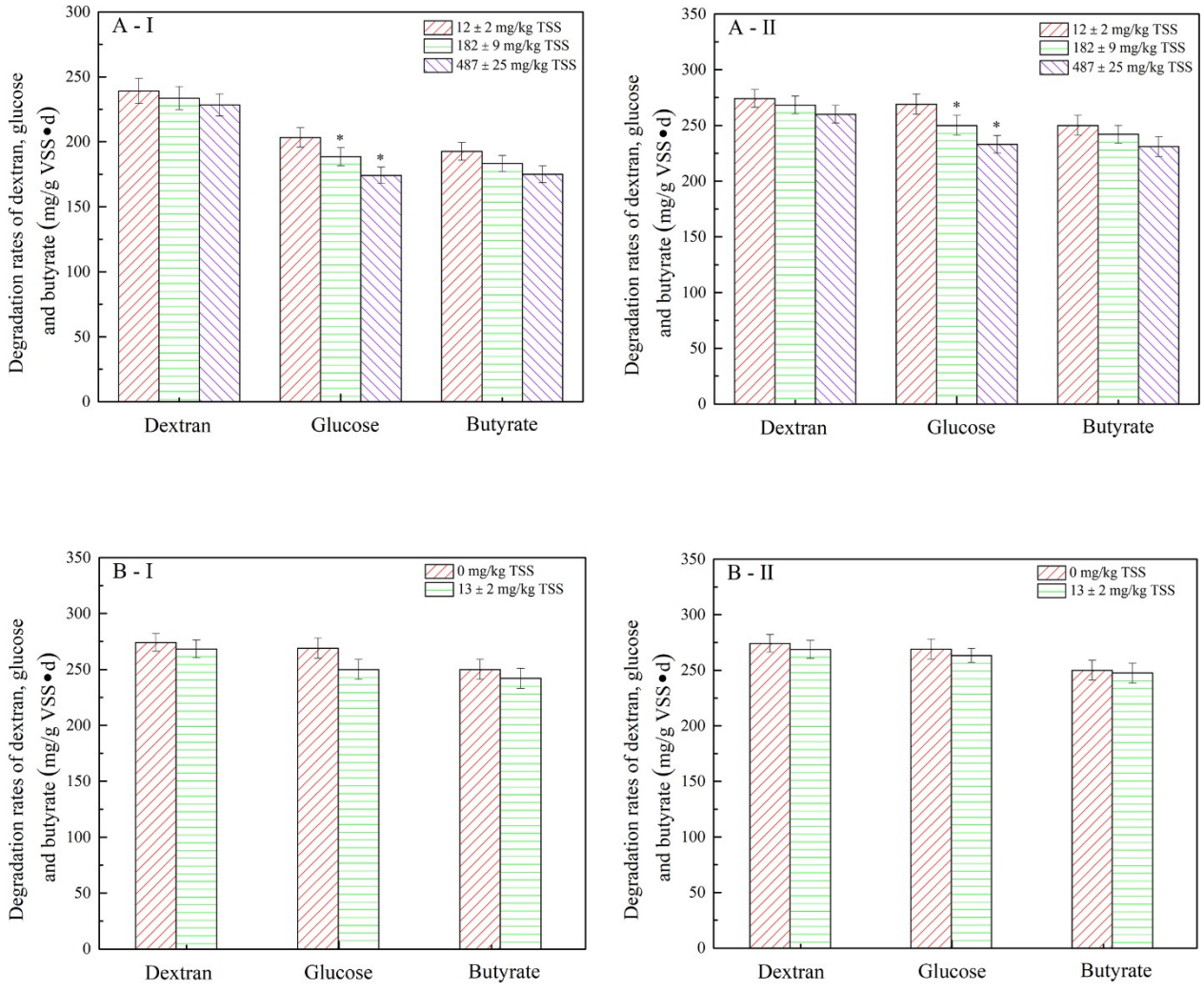
16 **Fig. 4. Cumulative hydrogen production from WAS dark fermentation in the presence of TCS at dif-**

17 **ferent levels (A) and correlation between TCS and the maximum hydrogen potential from model fit (B).**

18 **Symbols represent experimental measurements and lines represent model fits. Error bars represent**

19 **standard deviations of triplicate tests.**

20



21

22 **Fig. 5. Effect of TCS (A) and 2,4-DCP (B) on the specific degradation or consumption rate of model**
23 **compounds. A-I and B-I: Data were measured from alkaline fermentation (pH 10). A-II and B-II:**
24 **Data were measured from the reactors without pH control. Asterisks indicate statistical differences (p**
25 **< 0.05) from the control. Error bars represent standard deviations of triplicate tests.**

26

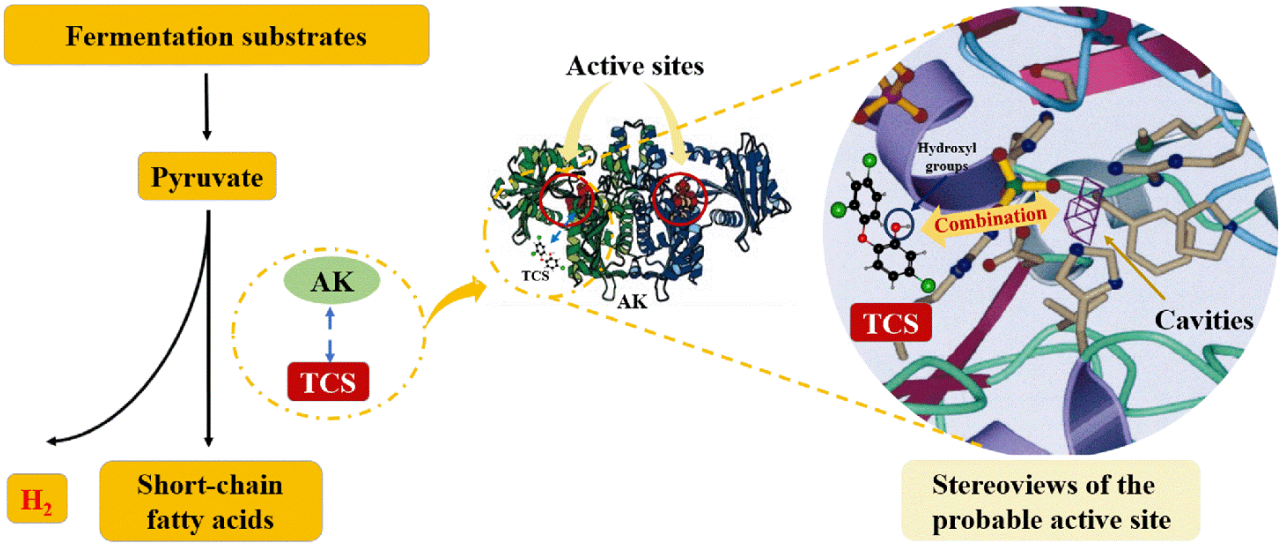


Fig. 6. Schematic diagram of how TCS affects acidogenesis process according to the reference.

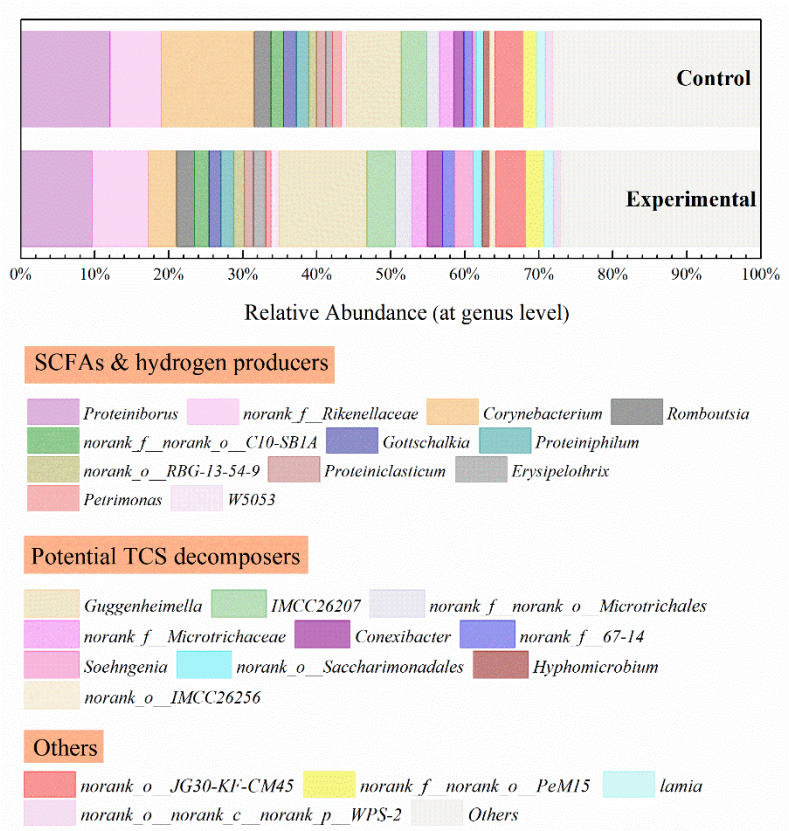


Fig. 7. The distribution of bacterial populations at genus level in the two long-term fermentation reactors.