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Triclosan Enhances Short-chain Fatty Acid Production from Sludge Fermentation by Elevating Transcriptional Activity of acidogenesis Bacteria

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ABSTRACT

Triclosan (TCS) is an emerging contaminant in waste activated sludge (WAS). However, the effects of TCS on WAS anaerobic fermentation is still unknown. Herein, we investigated the impacts of different TCS levels on the short-chain fatty acid (SCFA) accumulation. TCS at 100 mg/kg TSS (total suspended solids) significantly ($p<0.05$) enhanced SCFA production by 35.8 ± 4.1% though increasing the production of propionic and butyric acid, while the higher TCS level inhibited the SCFA production processes. The results of batch tests with real sludge showed that 100 mg/kg TSS TCS enhanced acidogenesis process and had no significant impact on other processes, while higher TCS level caused negative effects on acidogenesis, acetogenesis and methanogenesis. Generally, 2,4-dichlorophenol was considered as the main metabolic product of TCS under anaerobic condition. However, in this study, TCS was found to be converted into (Z)-4-(2,4-dichlorophenoxy)penta-1,3-dien-3-ol, 2,4-dichloro-1-(4-chloro-2-methoxyphenoxy)benzene and other substances, and its hypothetical metabolic pathway were reformulated. The microbial community structure in the WAS anaerobic fermentation system did not significantly change with TCS addition, but 100 mg/kg TSS TCS contributed to the modest enrichment of SCFA producers. Further analyses of key enzyme activity and abundance of enzyme-encoded genes revealed that TCS at 100 mg/kg TSS up-regulated the expression of enzyme-coding genes (i.e. butyrate kinase (BK) and oxaloacetic acid carboxylase (OAATC)), and this is the primary reason for the increased SCFA production. This work provides a new insight for pollutant effects in WAS anaerobic fermentation, which will help to reveal the impact of emerging contaminants or relearn the role of conventional pollutants.

Keywords: Waste activated sludge; Anaerobic fermentation; Emerging contaminants; Short-chain fatty acids; Transcription.

1. Introduction

Triclosan (TCS), a widely used low acute toxicity antimicrobial agent, serves as additives in personal care products (PCPs) and medical disinfectants [1]. The direct evidence of human widespread exposure to TCS is that this chemical is often detected in human urine. Each person is reported to consume 3-5 mg of TCS daily, and its annual consumption in the USA was estimated to be 357-594 tons [2]. Due to the difficult degradation of TCS in short term, increasing amounts of TCS were found in environment [3]. Thus, it is extremely necessary to have an in-depth understanding of the impact of TCS on various environments.

Wastewater treatment plants (WWTPs) are places where TCS in wastewater accumulates, also the last barriers before TCS enter the natural environments. Most of the TCS in wastewater was removed in WWTPs via precipitation, biosorption, or other biomass mediated processes [4, 5], and 76% of the influent TCS was adsorbed into the sludge [6]. In US, the highest concentration of TCS in secondary activated sludge in WWTPs reached 10.49 mg/kg dry solids [7]. In German, the concentrations found in sludge vary from 1-8 mg/kg dry solids [8]. In Canada, the anaerobically digested primary solids contained the high levels of TCS (median 22,700 ng/g) [9]. So far, more than 700 products have used TCS as an additive. As more and more TCS-based products are used, much higher concentrations of TCS in the waste activated sludge (WAS) can be expected in the immediate future. Additionally, the intercepted TCS in WAS might also bring risks to its subsequent treatment, which need urgent assessment.

Generally, anaerobic fermentation is selected to treat WAS to produce methane, and this process can also effectively kill pathogenic microorganisms and reduce the amount of sludge

[10-12]. Lately, growing attention has been focusing on the anaerobic fermentation of WAS to produce SCFAs [13, 14]. SCFAs can be exploited as raw materials for production of biodegradable plastics (e.g. polyhydroxyalkanoates) and many high value-added products such as pigments, cellulose acetate, paints, aspirin. Meanwhile, because the carbon source in wastewater cannot meet the requirement of biological nutrient removal, SCFAs could be employed as preferred extra carbon sources in this process [4, 15-18].

WAS anaerobic fermentation being a complex biological process with a variety of microorganisms involved (e.g. methanogenic *Archaea*, *Proteiniclasticum* sp. and *Sedimentibacter* sp.). TCS might inhibit these microorganisms and thus affect the anaerobic fermentation. Meanwhile, microorganisms in anaerobic fermentation system might degrade TCS [19] and affect the fate of TCS. Nowadays, increasing attention is paid to the impact of emerging contaminant on sludge anaerobic fermentation system [20], and the strategy for the PCPs removal from wastewater and sludge treatment have been studied [21]. However, the interaction between TCS and sludge anaerobic fermentation system is still largely unknown.

The work aims to investigate the effects of TCS on WAS anaerobic fermentation and reveal underlying mechanisms. The effects of different TCS levels on SCFA production were compared, and the mechanisms were probed from the genetic level. As far as we know, this is the first work clarifying the detailed effects of TCS on WAS anaerobic fermentation, which will fill the gap in the understanding of the impact of emerging contaminants (i.e. TCS) on sludge anaerobic fermentation process and may have important implications to sludge treatment.

2. Materials and methods

2.1 Source of WAS and triclosan

The WAS used in this paper was withdrawn from the secondary clarifier of a WWTP in Changsha, Hunan Province, China. The raw sludge was filtrated by 0.45 mm stainless-steel mesh, and concentrated by setting at 4°C for 24 h before use. The main characteristics of concentrated sludge are presented in Supporting Information. TCS (purity > 98%) was purchased from Aladdin, Shanghai, China.

2.2 The influence of different TCS level on SCFAs accumulation from WAS fermentation

Thirty sludge anaerobic fermentation reactors (1000 mL serum bottle) were divided into two groups (Group-A and Group-B), and each included 15 reactors. 600 mL of the concentrated sludge was feed to these reactors. The pH values in Group-A were not adjusted. As the alkaline condition has been reported to contribute to the production of SCFAs [22], Group-B was subjected to alkaline pretreatment (initial pH=10 was used in this work) by using 4 M sodium hydroxide (NaOH) and 4 M hydrochloric acid (HCl). Considering the continuous increase of TCS concentration in sludge in the future [23], external 0, 50, 100, 200, 500 mg/kg TSS of TCS was added (labeled as A_0, A_{50}, A_{100}, A_{200}, A_{500}, B_0, B_{50}, B_{100}, B_{200}, and B_{500}, respectively, three parallel samples were made for each set) to investigate its impacts on SCFAs accumulation under gradually increasing TCS level. And then, these reactors were aerated with nitrogen (N_2) for 3 min, sealed with rubber stopper, and put in an air-bath shaker (35 ± 2°C) at stirring speed of 160 rpm for 15 days.

2.3 Evaluation of TCS impact on different fermentation processes

Anaerobic fermentation of WAS usually simplified to the following five steps:

solubilization, hydrolysis, acidogenesis, acetogenesis and methanogenesis. The solubilization process is an efficiency-limit step in WAS anaerobic fermentation process [24]. The dissolution of polysaccharides and proteins is a typical solubilization process. Therefore, the susceptibility to TCS of WAS solubilization was commonly evaluated by analyzing the changes of soluble chemical oxygen demand (SCOD), soluble polysaccharide and soluble protein content in fermentation liquor. The susceptibility to TCS of others WAS anaerobic fermentation processes are usually evaluated by batch tests using suitable model substrates in real sludge [25]. In this test, 40 reproductive reactors (1000 mL serum bottle) were divided into four groups with ten in each (named Test-I, Test-II, Test-III and Test-IV). The experimental setting was detailed in Supporting Information. It is worth emphasizing that 0, 2.2 and 11 mg/L applied in this experiment are converted from 0, 100, 500 mg/kg TSS.

2.4 Long-term operated semi-continuous reactors

The alkaline pre-treated concentrated sludge (1600 mL) was fed to three reactors (2500 mL brown serum bottles, named Group-C), and 0, 100, 500 mg/kg TSS TCS was added to three reactors (labeled C_0, C_{100}, C_{500}) respectively to investigate the long-term impact of different TCS levels on microbial community and key enzyme activities. Since the maximum SCFA yield in Group-B appeared on the 8th day, the sludge retention time of Group-C was regulated as 8 days. During fermentation 200 mL fermentation liquid was withdrawn with syringes, and the corresponding concentrated sludge was injected into the reactor every day. Noteworthily, after 45 days of fermentation, the content of SCFAs were measured every 3 days. It took about 60 days until SCFA production achieved stability relatively. As another important indicator, the gaseous products such as methane and hydrogen of each reactor were

measured by the exhaust method. After SCFA production and gaseous products were relatively stable (the difference between adjacent two measurement values are less than 5%), the analyses of microbial community and enzyme activities were conducted.

2.5 Quantitative real-time PCR (qPCR) of the encoding gene of key enzyme

The total DNA in Group-C was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to instruction. Additional TCS affects butyrate kinase (BK) and oxaloacetic acid carboxylase (OAATC) activity signally, hence we quantified the encoding genes of them. The candidate primer sets were designed as follows: based on bacterial diversity at genus level based on 16S rDNA sequencing, we searched the encoding gene of BK and OAATC in the same genera in NCBI database as referenced gene sequences, and then designed primer set for each of them. The details of the referenced genes and the corresponding primers are listed in Table 1. To accurately quantify the mRNA of BK and OAATC genes, the designed primer sets were optimized by PCR: The primer sets were respectively subjected to PCR with the extracted total DNA as template, and the primer set with the best amplification efficiency was selected for real time qPCR. The optimized primer sets were used to quantify the coding gene of BK and OAATC with amplified DNA as a template, and the procedures and reaction mixture composition of the DNA qPCR are shown in Tables S1 and S2, respectively. All PCR assays were conducted using three replicates per sample.

Meanwhile, the total RNA in Group-C was extracted using Total RNA Isolation Kit (MoBio, Beijing, China) according to the manufacturer's instruction. To avoid contamination and increase the reliability of RNA quantitation, we used a one-step method to actualize a

rough comparison of mRNA of BK and OAATC. Quant One Step RT-qPCR Kit (SYBR Green) (TIANGEN, Beijing, China) was used for this purpose. The cDNA obtained from reverse transcription serves as a standard to calculate the copy numbers of mRNA of BK and OAATC. The procedures and reaction mixture composition of RNA qPCR is shown in Tables S3 and S4, respectively.

2.6 Data measurement

Total COD, soluble COD, TSS, and VSS were analyzed according to standard methods [26]. Soluble protein was detected by the bicinchoninic acid method with bovine serum albumin (BSA) as the standard, soluble polysaccharide was detected by the Lowry-Folin method with glucose as the standard. The total amino acid was determined by the ninhydrin method. The detailed procedures of SCFAs, TCS and its metabolite measurement were presented in Supporting Information. The activities of key enzymes (protease, α-glucosidase, acetate kinase (AK), butyrate kinase (BK), oxaloacetate transcarboxylase (OAATC) and coenzyme F_{420}) were measured by the reported method [27, 28]. Detection of methane fractions was consistent with literature [10]. The EEM fluorescence spectroscopy was obtained by fluorescence spectrophotometer (Hitachi F4600, Japan). Differences in the microbial community structure and microbial abundance among Group-C were analyzed by GENEWIZ (Suzhou, China).

2.7 Statistical analysis

All tests in this work were conducted in triplicate and the results were expressed as mean ± standard deviation. An analysis of variance by SPSS 23 software was used to evaluate the significance ($p < 0.05$) of results.

3 Results and discussion

3.1 SCFA production from sludge anaerobic fermentation in the presence of TCS

The exogenous addition of TCS has significant effect on the SCFAs accumulation. It should be stated that 500 mg/kg TSS TCS was equivalent to 14.6 mg COD/L based on conversion, so the effect of TCS conversion in this test was ignored in this work. Figures 1a and 1b showed the SCFAs accumulation in Group-A and Group-B. Group-A and Group-B have similar trends, from 1st day to 8th day and 6th day, respectively, the accumulated SCFAs increased with the fermentation time. Then the SCFAs content gradually decreased in the remaining fermentation time. In Group-A, the maximal yield of SCFAs in A_{200} was 399 mg COD/L, which is the lowest in the five tested TCS concentration, while the maximal yield of SCFAs in A_{50} was 899 mg COD/L, and the maximal yield of SCFAs in A_{100} reached 1111 mg COD/L, which is the highest in the five tested TCS concentration. These results suggested low concentration of TCS might enhance the capacity of WAS to produce SCFAs. Like Group-A, the highest maximal SCFA yield in Group-B was in B_{100} (4000 mg COD/L). The lowest maximal yield of SCFAs was in B_{200} (1880 mg COD/L). These results clearly indicated that the moderate TCS level (no more than 100 mg/kg TSS) significantly promoted SCFA production under different pH values ($p<0.05$). Appropriate addition of TCS could increase SCFA yield, but when TCS level further increased, the maximal yield of SCFAs drastically decreased. It is worth noting that 500 mg/kg TSS TCS has less effect on SCFA production. The reason for this phenomenon will be explained further below.

Total SCFAs are composed of acetic, propionic, iso-butyric, n-butyric, iso-valeric, and n-valeric acids. To clarify the reasons for the increase of SCFA production, the effect of TCS

on SCFA composition was further analyzed. Although acetic, n-butyric, iso-valeric, and n-valeric acids increased in 100 mg/kg TSS TCS reactors, the great promotion of propionic and n-butyric acid was the primary cause for the improvement of total SCFA production (Figures 1c and 1d). For example, the propionic acid production and total SCFA production in the 100 mg TCS/kg TSS reactors increased by 303 and 355 mg COD/L (A_{100}), and 673 and 1035 mg COD/L (B_{100}) respectively contrast to that in 0 mg/kg TSS TCS reactors, suggesting that the increment of propionic acid accounted for 85.4% and 65.0% to total SCFA promotion, respectively. And the increment of n-butyric acid accounted for 12.4% and 20.7% to total SCFA promotion, respectively. Similar results have been also observed in other reactors. With TCS addition from 0 to 100 mg/kg TSS, the fraction of propionic acid increased from 55.4 to 65% (Group-A), and from 16.9 to 29.6% (Group-B), respectively. And with the augment of additional TCS from 0 to 100 mg TCS/kg TSS, the fraction of n-butyric acid increased from 3 to 6% (Group-A), and from 5.7 to 9.6% (Group-B), respectively. All above results demonstrated that TCS content affects SCFA accumulation, especially propionic and n-butyric acid production.

To understand the effect of TCS metabolites on experimental results, the concentration of TCS in the A_{500} was measured for 15 consecutive days. About 77% of the original additional TCS was detected after 15 days of metabolism (Figure 2a). The degradation products of TCS were detected by LC-MS/MS. Thirteen product ions at m/z 161, m/z 261, m/z 245, m/z 306, m/z 320, m/z 336, m/z 304, m/z 283, m/z 237, m/z 221 m/z 274 m/z 239 and m/z 233 were detected, and the degradation reaction pathway was deduced based on products and previous reports [29-31]. Several possible degradation pathways for TCS could

be speculated, and the result was presented in Figure 2b. In pathway I, TCS is produced 2,4-dichlorophenol by hydrolysis. In pathway II, TCS transforms (1E,3Z)-4-(2,4-dichlorophenoxy)penta-1,3-diene-1,3-diol and (Z)-4-(2,4-dichlorophenoxy)penta-1,3-dien-3-ol by dechlorination and chain opening, dehydroxylation. In pathway III, TCS is metabolized to 2-chloro-5-(2,4-dichlorophenoxy)benzene-1,4-diol, 2-chloro-5-(2,4-dichlorophenoxy)-4-methoxyphenol and 3,5-dichloro-2-(4-chloro-5-hydroxy-2-methoxyphenoxy)phenol in sequence. In pathway IV, TCS is first methylated to produce 2,4-dichloro-1-(4-chloro-2-methoxyphenoxy)benzene, which is then converted to 3,7-dichloro-1-methoxydibenzo[b,e][1,4]dioxine. In pathway V, TCS is metabolized to 2,4-dichloro-1-(4-chlorophenoxy)benzene and 2-(2,4-dichlorophenoxy)malonaldehyde in sequence. In pathway VI, TCS is converted to 5-chloro-2-(2-hydroxyphenoxy)phenol and 2-(4-chlorophenoxy)phenol in turn. In summary, TCS has a variety of metabolic pathways in anaerobic sludge, including but not limited to processes such as dehalogenation, hydroxylation, and methylation, which indicated that TCS might be involved in the biological processes of sludge anaerobic fermentation. It is essential to investigate the effects of TCS on different processes of sludge anaerobic fermentation.

3.2 Dose-dependent effect of TCS on different fermentation processes

The solubilization is generally considered to be a rate-limit process for sludge resource utilization [32, 33], which could affect the accumulation of SCFAs. The results of dissolved organic matter (SCOD, soluble proteins and soluble polysaccharides) measurement in Group-A and Group-B have no significant change (Figure S1). The EEM fluorescence

spectroscopy suggested there was no structural change in fermentation liquid. Above experimental results indicated that the addition of TCS had no effect on the solubilization process. It is notable that by monitoring the soluble protein/total protein ratio (usually as an indicator of cell rupture) in the reactor (Figure S3), we found that the cells in the A_{500} are more susceptible to disrupt. Cell lysis releases more matrix for the synthesis of SCFAs. This could explain why SCFA production of the 500 mg/kg TSS TCS sludge reactors was higher than that of the 200 mg/kg TSS TCS sludge reactors.

Macromolecular substances can only be further utilized by microorganisms in sludge after they are hydrolyzed into small molecules [34, 35]. BSA and dextran were used as model substrates in this test. The degradation ratio of BSA and dextran increased from 55% and 72% to 67.4% and 87% from the 1st day to the 5th day, respectively (Figure S4 and Table 2). Furthermore, the degradation of BSA and dextran in fermentation reactors with different TCS levels showed no significant difference ($p>0.05$), suggesting TCS did not affect the hydrolysis.

Acetic acid is one of the major products in acidogenesis and acetogenesis processes among SCFAs, which could serve as main substrate in the methanogenesis process [36]. Other SCFAs like propionic acid (one of the other main products in acidogenesis), butyric acid and valeric acids are also produced in acidogenesis process [37]. Glucose and amino acids as model substances can indicate the impact of TCS in the acidogenesis process. The 2.2 mg/L TCS significantly improved the degradation ratio of glucose ($p<0.01$). For example, on the 1st day of fermentation, the degradation ratio of glucose in the 2.2 mg/L TCS sludge reactor increased from 74% to 89% compared with the 0 mg/L sludge reactor, while the

glucose degradation ratio in the 11 mg/L TCS sludge reactor dropped to 45%, and the similar results also appeared in other time of fermentation. The 100 mg/kg TSS TCS also enhanced the degradation ratio of compound amino acid ($p<0.01$), suggesting that the presence of 2.2 mg/L TCS enhanced acidogenesis, and 11 mg/L TCS inhibited it.

Before being used to produce methane, other SCFAs need to be converted to acetic acid via microorganisms related to acetogenesis. The degradation of sodium butyrate and the productions of acetic acid were used to clarify the effect of TCS on acetogenesis process. It can be seen from Table 2 that butyric acid content did not change much with TCS increasing in same time ($p>0.05$), but 11 mg/L TCS obviously inhibited the production of acetic acid. However, in fact, there is no significant change in the total peak area of the gas chromatographic analysis ($p>0.05$), indicating that although 11 mg/L TCS reduced the yield of acetic acid, sodium butyrate was converted into other substances. All these results indicated that TCS did not affect the decomposition of acetic acid precursors, but high concentrations of TCS affected the synthesis of acetic acid.

The acetic acid from the acidogenesis and acetogenesis process can be consumed for the methanogenesis process, therefore, the effect of different TCS levels on this process was also assessed. From Table 2, the degradation ratio of acetic acid does not change with the increasing TCS at any time ($p>0.05$), but 11 mg/L TCS obvious inhibited the production of methane ($p<0.05$). The results suggested that high concentration of TCS affected methanogenesis process by inhibiting the synthesis of methane.

In summary, moderate TCS addition (i.e. 2.2 mg/L, equivalent to 100 mg/kg TSS in other tests) did not affect solubilization, hydrolysis, acetogenesis and methanogenesis. The

impact of TCS on acidogenesis is dose-dependent, 2.2 mg/L TCS enhanced this process, but 11 mg/L TCS (equivalent to 500 mg/kg TSS in other tests) displayed the opposite effect. In addition, 11 mg/L TCS also had adverse effects on the synthesis of acetic acid in acetogenesis and methanogenesis process. It can be therefore understood why the 2.2 mg/L TCS caused greater maximum SCFA yield largely.

3.3 Comparison of microbial structure and abundance under different TCS level

Microbial community structure and functional microbial abundance are closely relevant to SCFAs accumulation [38]. Hence, the microbial communities among Group-C were analyzed. The Illumina Hiseq results of 16S rDNA showed that the bacterial communities in the Group-C have no obvious difference. The details of the OTUs are further demonstrated by the Venn diagram (Figure S5). The results indicated that the addition of TCS did not affect the microbial structure greatly. As far as we know, microbial structure in sludge anaerobic fermentation system is relatively stable, and small amount of exogenous organic matter will not significantly affect it [4, 17].

At the phylum level, mostly microorganisms were affiliated to the following five phyla: Proteobacteria, Bacteroidetes, Firmicutes, Acidobacteria and Chloroflexi (Figure 3a). Under anaerobic conditions, many microbes belonging to these phyla could decompose a variety complex organic compounds for SCFA production, such as polysaccharides and proteins [39]. Among them, Firmicutes and Proteobacteria were the predominant microorganisms in all long-term reactors. Many bacteria affiliated to Proteobacteria were reported as SCFAs producers, and be able to produce acetic acid as their major metabolic products under acidogenesis process, while some microbes of Firmicutes were acetic acid consumers [40].

With the increase of additional TCS content, the abundance of Firmicutes slightly increased but that of other phyla did not significantly change.

The evolution of microorganisms in Group-C was illustrated in Figure S6. Several types of SCFAs producers such as *Sedimentibacter* sp., *Lentimicrobium* sp., *Proteocatella* sp., *Tissierella* sp., *Christensenellaceae* sp., and *Macellibacteroides* sp. were detected in both reactors (Figure 4a) [41]. The *Proteocatella* sp. was an anaerobically fermentative bacterium with acetate, butyrate and ethanol as the main fermentative end products [42]. The abundance of *Proteocatella* was 4.49% of the total bacterial sequences in the C_0, whereas the corresponding value was 4.72% and 7.03% in C_{100} and C_{500}, respectively. The *Lentimicrobium* is a strictly anaerobic bacterium, and the major fermentative end products of substrate were acetate, malate, propionate, formate and hydrogen [43]. The abundance of *Lentimicrobium* was accounted to 8.34% of the total microorganisms in the C_0, whereas the corresponding value was 10.07% and 9.51% in C_{100} and C_{500}, respectively. Moreover, total abundance of these SCFAs producers in C_0, C_{100} and C_{500} did not show much difference (11.97%, 13.01% and 13.72%, respectively). In Section 3.2, we know that the 100 mg/kg TSS TCS promoted the production of propionic acid and butyric acid, and we found that the abundance of the genera with propionic acid as main metabolites, such as *Veillonellaceae* and *Thermomonas*, were increased in C_{100}, which indicates that the increase in the production of propionic acid and butyric acid might be caused by the increase in microorganisms abundance [44]. The abundance of SCFA producers had different changes under the stimulation of TCS. In addition to the microorganisms described above, some producer's abundance had no change, even decreased, so the change in total SCFA producer's abundance is not significant.

From Figure 4a, it can be found that some microorganisms with specific function, were detected at a substantial level. The *Pseudomonas* sp. was be capable of utilizing some organic contaminants as nutrients for its own growth, like hydrophobic polycyclic aromatic hydrocarbons (HAPs) [45], the abundance of *Pseudomonas* was 0.26% of the total bacterial sequences in C_0, whereas the corresponding value was 0.59% and 1.37% in the C_{100} and C_{500}, respectively. *Novosphingobium* sp. was reported to be capable of degrading some aromatic contaminants, like nitrobenzene, aniline and phenanthrene [46], and its relative abundances in C_{500} was higher than that in C_0, the abundance of *Novosphingobium* was 0.17% of the total bacterial sequences in C_0, whereas the corresponding value was 0.21% and 0.30% in C_{100} and C_{500}, respectively, suggesting that *Novosphingobium* sp. and *Pseudomonas* sp. might be potential microbes for TCS degradation. The genera of *Saprospiraceae*, *Ottowia* and *Thermomonas* were detected in both reactors. It is reported that these microorganisms are capable of producing and secreting quantities of extracellular enzymes, like phosphatase and proteases [47]. These extracellular enzymes are key enzymes for degrading solubilized substrates for subsequent acidification [48, 49].

In summary, the addition of TCS did not cause changes in the microbial community structure of sludge anaerobic fermentation system but affected the abundance of SCFAs producers and potential TCS decomposers in it.

3.4 TCS affect transcriptional levels of key enzyme-coding genes for acidogenesis

It is known that the SCFA production from WAS anaerobic fermentation is chiefly attributed to biological effects, so detection of key enzyme activities is a crucial way to monitor related biological process in anaerobic fermentation reactors [24]. Six key enzymes

(protease, α-glucosidase, AK, BK, OAATC and coenzyme F_{420}) involved in sludge anaerobic fermentation were selected in this work. Protease and α-glucosidase could decompose protein and polysaccharide into amino acids and monosaccharide in the hydrolysis process, respectively. Subsequently, the hydrolyzed products are respectively transformed into acetic acid and butyric acid by the AK and BK. OAATC is vital in the propionic acid production process while coenzyme F_{420} play a crucial role in methanogenesis (Figure S7a) [28, 50].

500 mg/kg TSS TCS inhibited the activity of coenzyme F_{420} (Figure S7b), which may be one reason why the accumulated SCFAs in A_{500} and B_{500} were more than that in A_{200} and B_{200}, and explained why the 11 mg/L TCS inhibited the methanogenesis process. In addition, the decrease in AK activity in C_{500} is also consistent with the results in Section 3.2, and other key enzyme activities in C_{500} were unaffected.

Consider the concentration of TCS will not reach such a significant level (500 mg/kg TSS) in short-term, and TCS at 100 mg/kg TSS markedly affected the sludge anaerobic fermentation system. To revealing the reason of 100 mg/kg TSS TCS enhanced SCFA production. The relative key enzyme activities in C_0 and C_{100} were further compared. There had no significant change in relative activities of protease, α-glucosidase and AK. The relative enzyme activities of BK and OAATC in the C_{100} was markedly higher than that in the C_0. Previous studies considered that changes in enzyme activity as a result of an increase in producers' abundance, further lead to an increase in SCFA production [4, 51]. In order to verify the above inference, we assumed: 100 mg/kg TSS TCS stimulates the enrichment of the SCFAs producer and thus causes the increase in the activity of key enzymes (i.e. BK & OAATC). If the hypothesis is true, then the encoding genes abundance of BK and OAATC in

C_{100} should be higher than that in C_0. It has been reported that 20 and 50 mg/kg dry sludge sulfadiazine could increase the copy number of key enzymes (i.e. protease, AK, and α-glucosidase) [52]. The encoding genes abundance of BK and OAATC were further confirmed by real-time quantitative PCR using the optimized primer sets 'BK-7' and 'OAATC-2' (Table 1), respectively. Interestingly, there was no significant change in the DNA copy number of the encoding genes of BK and OAATC in C_0 and C_{100} ($p>0.05$) (Figure 4b). To verify if the abundance of other functional genes has changed, the classification and abundance of functional genes in each reactor were compared, the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) results showed that there was almost no change in the type and abundance of 'COG Function Classification' in Group-C (Figure 3b). Obviously, the experiment results overturned our previous assumption, the enrichment of SCFA producers was not the cause of the increase in the activity of key enzymes.

How exactly does TCS at 100 mg/kg TSS level increase the amounts of BK and OAATC? The up-regulation of the transcriptional level of the enzyme-coding gene can lead to higher enzyme activity when the gene abundance is constant. Most of the studies on sludge anaerobic fermentation did not further discuss its influence on gene [53], although the regulation of gene transcription level is a widespread phenomenon [54]. The qPCR of mRNA showed that the mRNA abundance of BK and OAATC genes in C_{100} was greater than that in C_0 (Figure 4c), this may be the main reason for the increased activity of key enzymes. SPSS analysis further indicated that the changes in the two enzyme-coding genes' transcriptional level were significantly correlated with those of enzyme activities ($p<0.01$). Therefore, the TCS at 100

mg/kg TSS level stimulated the transcription of the key enzyme-encoding genes in microorganisms involved in acidogenesis process, and promoted the key enzymes' activities, which eventually increased the SCFA production in sludge fermentation liquid.

3.5 Implications

TCS has become an emerging contaminant and attracted increasing concerns in recent years. This work revealed the interaction between TCS and the WAS anaerobic fermentation system. Although TCS usually was thought as inhibitor of biological process, our work indicated that the existence of TCS in a suitable concentration range could enhance SCFAs accumulation from WAS, which was formerly existed in WAS but unrecognized before. With the growing worldwide energy crisis, WWTPs are increasingly recognized as places for energy recovery rather than waste removal [55]. Figure 5 shows a typical two-stage process configuration for a typical wastewater treatment plant. In this type of WWTPs, most organic matter in influent is adsorbed/stored by activated sludge, the WAS was subsequently sent to the anaerobic fermenter for bioenergy recovery.

It should be noticed that increasing TCS level do not affect solubilization, hydrolysis and acetogenesis process, however, acidogenesis and methanogenesis are sensitive to the dose of TCS, both of which are inhibited by high TCS level. It is worth emphasizing that 100 mg/kg TSS TCS could stimulate the system to produce the maximum SCFAs by enhancing the transcription levels of the key enzyme-coding genes involved in the acidogenesis process. In summary, it can be concluded that although the current actual concentration of TCS does not reach the level to endanger the WAS anaerobic fermentation system, considering that TCS is not easy to degrade, it will pose a threat to the aquatic environment after treatment. It is

reported that TCS is easily enriched in fish tissues [56], which may pose a threat to human health through the food chain. To removal TCS from water, various treatment technologies have been investigated, including ozonation, potassium permanganate oxidation, anodic oxidation, Fenton and electro-Fenton oxidation, photolysis, nanofiltration, and thermally activated persulfate oxidation [57-64]. Decision-makers should consider the pros and cons of these approaches and choose the most appropriate approach in WWTPs.

Another finding of this work was 'cut off' the direct link between functional microorganism abundance and enzyme activity, and revealing the promoting effect of TCS on gene transcription. The chronic exposure to an environmentally relevant TCS concentration could induce persistent TCS resistance [65], what the effect of more TCS on various microbes, by promoting gene transcription? This needs to be answered in subsequent studies. It should also be emphasized that a considerable part of TCS was not degraded in WAS anaerobic fermentation process, the remaining TCS would ineluctably enter the environment again, which pose a potential risk to the environment. Thus, a pretreatment that could effectively promote TCS degradation in WAS and does not adversely affect sludge anaerobic fermentation is required.

4. Conclusion

This study assessed the effect of TCS on SCFA production from WAS anaerobic fermentation. TCS in proper concentration (less than 100mg/kg TSS) enhanced SCFA production by stimulating the synthesis of propionic acid and butyric acid on acidogenesis process, but higher TCS level inhibited some production processes. In addition, we found that the degradation pathway of TCS under anaerobic conditions is more diverse than previous understanding, and TCS produced a variety of metabolites (e.g. (Z)-4-(2,4-dichlorophenoxy)penta-1,3-dien-3-ol and 2,4-dichloro-1-(4-chloro-2-methoxyphenoxy)benzene). The microbial community structure in anaerobic fermentation system did not differ greatly under different TCS levels, but 100 mg/kg TSS TCS contributed to the slight SCFA producer's abundance increasement. Interestingly, increase of SCFAs producer's abundance was not the reason of the augment in key enzyme (i.e. BK & OAATC) activity involved in acidogenesis, as the gene abundance of BK and OAATC did not change significantly. Finally, combined the above results with the quantitative analysis of mRNA to arrive at the following conclusions: The moderate additional of TCS (i.e. 100 mg/kg TSS) can up-regulated the transcription level of enzyme-coding gene on acidogenesis process, resulting in changes in WAS anaerobic fermentation.

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REFERENCES

1. Wang, S., K. Poon, and Z. Cai, *Removal and metabolism of triclosan by three different microalgal species in aquatic environment.* J. Hazard. Mater., 2018. **342**: p. 643-650.
2. Anger, C.T., et al., *Quantification of Triclosan, Chlorinated Triclosan Derivatives, and their Dioxin Photoproducts in Lacustrine Sediment Cores.* Environ. Sci. Technol, 2013. **47**(4): p. 1833-1843.
3. Xin, X., et al., *Insights into Long-Term Toxicity of Triclosan to Freshwater Green Algae in Lake Erie.* Environ. Sci. Technol, 2019. **53**(4): p. 2189-2198.
4. Wang, Y., et al., *Triclocarban enhances short-chain fatty acids production from anaerobic fermentation of waste activated sludge.* Water Res., 2017. **127**: p. 150-161.
5. Sun, Y.X., et al., *Hydrocyclone-induced pretreatment for sludge solubilization to enhance anaerobic digestion.* Chemical Engineering Journal, 2019. **374**: p. 1364-1372.
6. Heidler, J. and R.U. Halden, *Mass balance assessment of triclosan removal during conventional sewage treatment.* Chemosphere, 2007. **66**(2): p. 362-369.
7. Kinney, C.A., et al., *Survey of Organic Wastewater Contaminants in Biosolids Destined for Land Application.* Environ. Sci. Technol, 2006. **40**(23): p. 7207-7215.
8. Bester, K., *Triclosan in a sewage treatment process—balances and monitoring data.* Water Res., 2003. **37**(16): p. 3891-3896.
9. Guerra, P., et al., *Occurrence and removal of triclosan in Canadian wastewater systems.* Environmental science and pollution research international, 2019.
10. Wang, D., et al., *How Does Poly(hydroxyalkanoate) Affect Methane Production from*

the Anaerobic Digestion of Waste-Activated Sludge? Environ. Sci. Technol, 2015. **49**(20): p. 12253-12262.

11. Yan, W.W., et al., *The interactive effects of ammonia and carbon nanotube on anaerobic digestion.* Chemical Engineering Journal, 2019. **372**: p. 332-340.
12. Zhang, L., K.C. Loh, and J.X. Zhang, *Jointly reducing antibiotic resistance genes and improving methane yield in anaerobic digestion of chicken manure by feedstock microwave pretreatment and activated carbon supplementation.* Chemical Engineering Journal, 2019. **372**: p. 815-824.
13. Saha, S., et al., *Microbial acclimatization to lipidic-waste facilitates the efficacy of acidogenic fermentation.* Chemical Engineering Journal, 2019. **358**: p. 188-196.
14. Dahiya, S. and S.V. Mohan, *Selective control of volatile fatty acids production from food waste by regulating biosystem buffering: A comprehensive study.* Chemical Engineering Journal, 2019. **357**: p. 787-801.
15. Jiang, Y., Y. Chen, and X. Zheng, *Efficient Polyhydroxyalkanoates Production from a Waste-Activated Sludge Alkaline Fermentation Liquid by Activated Sludge Submitted to the Aerobic Feeding and Discharge Process.* Environ. Sci. Technol, 2009. **43**(20): p. 7734-7741.
16. Yuan, H., et al., *Improved bioproduction of short-chain fatty acids (SCFAs) from excess sludge under alkaline conditions.* Environ. Sci. Technol, 2006. **40**(6): p. 2025.
17. Luo, J., Y. Chen, and L. Feng, *Polycyclic Aromatic Hydrocarbon Affects Acetic Acid Production during Anaerobic Fermentation of Waste Activated Sludge by Altering Activity and Viability of Acetogen.* Environ. Sci. Technol, 2016. **50**(13): p. 6921-6929.

18. Lin, C.B., et al., *Enhanced biogas production and biodegradation of phenanthrene in wastewater sludge treated anaerobic digestion reactors fitted with a bioelectrode system.* Chemical Engineering Journal, 2019. **365**: p. 1-9.

19. Taweetanawanit, P., T. Ratpukdi, and S. Siripattanakul-Ratpukdi, *Performance and kinetics of triclocarban removal by entrapped Pseudomonas fluorescens strain MC46.* Bioresour. Technol, 2019. **274**: p. 113-119.

20. Zhao, L., et al., *Effects of individual and combined zinc oxide nanoparticle, norfloxacin, and sulfamethazine contamination on sludge anaerobic digestion.* Bioresour. Technol, 2019. **273**: p. 454-461.

21. Biel-Maeso, M., C. Corada-Fernández, and P.A. Lara-Martín, *Removal of personal care products (PCPs) in wastewater and sludge treatment and their occurrence in receiving soils.* Water Res., 2019. **150**: p. 129-139.

22. Chen, Y., et al., *Hydrolysis and acidification of waste activated sludge at different pHs.* Water Res., 2007. **41**(3): p. 683-689.

23. Ma, Z., H. Liu, and H. Yu, *Triclosan affects Ca2+ regulatory module and musculature development in skeletal myocyte during early life stage of zebrafish (Danio rerio).* Environmental Science & Technology, 2019.

24. McCarty, P.L. and D.P. Smith, *Anaerobic wastewater treatment.* Environ. Sci. Technol, 1986. **20**(12): p. 1200-1206.

25. Xu, Q., et al., *Understanding and mitigating the toxicity of cadmium to the anaerobic fermentation of waste activated sludge.* Water Res., 2017. **124**: p. 269-279.

26. *Standard Methods for the Examination of Water and Wastewater, 20th ed*. 1998,

Washington, DC: American Public Health Association (APHA), American Water Works Association, and Water Environment Federation.

27. Goel, R., et al., *Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor.* Water Res., 1998. **32**(7): p. 2081-2088.

28. Mu, H. and Y. Chen, *Long-term effect of ZnO nanoparticles on waste activated sludge anaerobic digestion.* Water Res., 2011. **45**(17): p. 5612-5620.

29. Zhu, L., et al., *Electrochemical simulation of triclosan metabolism and toxicological evaluation.* Sci. Total Environ., 2018. **622-623**: p. 1193-1201.

30. Azarpira, H., et al., *Photo-catalytic degradation of triclosan with UV/iodide/ZnO process: Performance, kinetic, degradation pathway, energy consumption and toxicology.* J. Photoch. Photobio. A, 2019. **371**: p. 423-432.

31. Li, Q., et al., *Degradation of triclosan by chlorine dioxide: Reaction mechanism,2,4-dichlorophenol accumulation and toxicity evaluation.* Chemosphere, 2018. **207**: p. 449-456.

32. Appels, L., et al., *Principles and potential of the anaerobic digestion of waste-activated sludge.* Prog. Energ. Combust., 2008. **34**(6): p. 755-781.

33. Li, W., et al., *Co-treatment of potassium ferrate and ultrasonication enhances degradability and dewaterability of waste activated sludge.* Chemical Engineering Journal, 2019. **361**: p. 148-155.

34. Li, B. and T. Zhang, *Biodegradation and Adsorption of Antibiotics in the Activated Sludge Process.* Environ. Sci. Technol, 2010. **44**(9): p. 3468-3473.

35. Madigou, C., et al., *Ecological consequences of abrupt temperature changes in*

anaerobic digesters. Chemical Engineering Journal, 2019. **361**: p. 266-277.

36. Zhang, D., et al., *New Sludge Pretreatment Method to Improve Methane Production in Waste Activated Sludge Digestion.* Environ. Sci. Technol, 2010. **44**(12): p. 4802-4808.

37. Zheng, X., et al., *Pyrosequencing Reveals the Key Microorganisms Involved in Sludge Alkaline Fermentation for Efficient Short-Chain Fatty Acids Production.* Environ. Sci. Technol, 2013. **47**(9): p. 4262-4268.

38. Pan, X.F., et al., *Impact of nano zero valent iron on tetracycline degradation and microbial community succession during anaerobic digestion.* Chemical Engineering Journal, 2019. **359**: p. 662-671.

39. Boetius, A., et al., *A marine microbial consortium apparently mediating anaerobic oxidation of methane.* Nature, 2000. **407**: p. 623.

40. Lu, L., D. Xing, and N. Ren, *Pyrosequencing reveals highly diverse microbial communities in microbial electrolysis cells involved in enhanced H2 production from waste activated sludge.* Water Res., 2012. **46**(7): p. 2425-2434.

41. Wang, D., et al., *Short-Chain Fatty Acid Production from Different Biological Phosphorus Removal Sludges: The Influences of PHA and Gram-Staining Bacteria.* Environ. Sci. Technol, 2013. **47**(6): p. 2688-2695.

42. Li, L., et al., *Efficient Volatile Fatty Acids Production from Waste Activated Sludge after Ferrate Pretreatment with Alkaline Environment and the Responding Microbial Community Shift.* ACS Sustain. Chem. Eng., 2018. **6**(12): p. 16819-16827.

43. Sun, L.W., et al., *Lentimicrobium saccharophilum gen. nov., sp nov., a strictly anaerobic bacterium representing a new family in the phylum Bacteroidetes, and*

proposal of Lentimicrobiaceae fam. nov. Int. J. Syst. Evol. Micr., 2016. **66**: p. 2635-2642.

44. Spirito, C.M., et al., *Chain elongation in anaerobic reactor microbiomes to recover resources from waste.* Curr. Opin. Chem. Eng., 2014. **27**: p. 115-122.

45. Yuan, Y., et al., *Short-chain fatty acids production and microbial community in sludge alkaline fermentation: Long-term effect of temperature.* Bioresour. Technol, 2016. **211**: p. 685-690.

46. Cecil, J.H., et al., *Rapid, Parallel Identification of Catabolism Pathways of Lignin-Derived Aromatic Compounds in Novosphingobium aromaticivorans.* Appl. Environ. Microbiol., 2018. **84**(22): p. e01185-18.

47. Ma, J., et al., *Long-term investigation of a novel electrochemical membrane bioreactor for low-strength municipal wastewater treatment.* Water Res., 2015. **78**: p. 98-110.

48. McIlroy, S.J., et al., *Identification of active denitrifiers in full-scale nutrient removal wastewater treatment systems.* Environ. Microbiol., 2016. **18**(1): p. 50-64.

49. Huang, Z., et al., *Nitrification/denitrification shaped the mercury-oxidizing microbial community for simultaneous Hg0 and NO removal.* Bioresour. Technol, 2019. **274**: p. 18-24.

50. Liu, K., et al., *Effect of Humic Acids with Different Characteristics on Fermentative Short-Chain Fatty Acids Production from Waste Activated Sludge.* Environ. Sci. Technol, 2015. **49**(8): p. 4929-4936.

51. Liu, X., et al., *Unveiling the mechanisms of how cationic polyacrylamide affects*

short-chain fatty acids accumulation during long-term anaerobic fermentation of waste activated sludge. Water Res., 2019. **155**: p. 142-151.

52. Xie, J., et al., *Influence of sulfadiazine on anaerobic fermentation of waste activated sludge for volatile fatty acids production: Focusing on microbial responses.* Chemosphere, 2019. **219**: p. 305-312.

53. Wang, X., et al., *Stepwise pH control to promote synergy of chemical and biological processes for augmenting short-chain fatty acid production from anaerobic sludge fermentation.* Water Res., 2019. **155**: p. 193-203.

54. Harbison, C.T., et al., *Transcriptional regulatory code of a eukaryotic genome.* Nature, 2004. **431**: p. 99.

55. Feng, L., Y. Chen, and X. Zheng, *Enhancement of Waste Activated Sludge Protein Conversion and Volatile Fatty Acids Accumulation during Waste Activated Sludge Anaerobic Fermentation by Carbohydrate Substrate Addition: The Effect of pH.* Environ. Sci. Technol, 2009. **43**(12): p. 4373-4380.

56. Yao, L., et al., *Bioaccumulation and risks of 24 personal care products in plasma of wild fish from the Yangtze River, China.* Science of The Total Environment, 2019. **665**: p. 810-819.

57. Chen, J., et al., *Oxidative degradation of triclosan by potassium permanganate: Kinetics, degradation products, reaction mechanism, and toxicity evaluation.* Water Res., 2016. **103**: p. 215-223.

58. Gao, H., et al., *Sulfate radicals induced degradation of Triclosan in thermally activated persulfate system.* Chem. Eng. J., 2016. **306**: p. 522-530.

59. Ogutverici, A., et al., *Triclosan removal by NF from a real drinking water source – Effect of natural organic matter.* Chem. Eng. J., 2016. **283**: p. 330-337.

60. Suarez, S., et al., *Kinetics of triclosan oxidation by aqueous ozone and consequent loss of antibacterial activity: Relevance to municipal wastewater ozonation.* Water Res., 2007. **41**(12): p. 2481-2490.

61. Aaron, J.-J., *Advanced Oxidation Processes in Water/Wastewater Treatment: Principles and Applications. A Review AU - Oturan, Mehmet A.* Crit. Rev. Env. Sci. Tec., 2014. **44**(23): p. 2577-2641.

62. Wang, J. and J. Farrell, *Electrochemical Inactivation of Triclosan with Boron Doped Diamond Film Electrodes.* Environ. Sci. Technol, 2004. **38**(19): p. 5232-5237.

63. Mao, R., et al., *Dechlorination of triclosan by enhanced atomic hydrogen-mediated electrochemical reduction: Kinetics, mechanism, and toxicity assessment.* Appl. Catal. B-Environ., 2019. **241**: p. 120-129.

64. Liu, Y., et al., *Heterogeneous Fenton-like catalyst for treatment of rhamnolipid-solubilized hexadecane wastewater.* Chemosphere, 2019. **236**: p. 124387.

65. Li, M., et al., *Chronic Exposure to an Environmentally Relevant Triclosan Concentration Induces Persistent Triclosan Resistance but Reversible Antibiotic Tolerance in Escherichia coli.* Environ. Sci. Technol, 2019. **53**(6): p. 3277-3286.

Table 1 Candidate primer sets used for qPCR. Rows with emphasis are optimized primer set

Number	Label	NCBI Gene ID	Abundance ranking	Primer F	Primer R
1	OAATC-1	35558611	62	GCTGGTGCGGACATTATTGA	CCATCTGGCTCATAGGAGTAAC
2	OAATC-2	5107808	30	CGACGATGTGGTGGAGAAGT	GCTGGTGGTGTAGGAGATGG
3	OAATC-3	28545948	30	TTGTCCTCGGTGTCTATTCTCA	AGTAGGCGGCAATCTCTTCC
4	OAATC-4	28544883	30	GAAGTGCTGGCGGAGATTC	CCTCGGTGGTGATGGTCTT
5	OAATC-5	36010628	30	AAGGAGATGGAGGCGATGG	TGGTTGGCGAGGTTGGAA
6	BK-1	5187685	120 & 189	GCTTCTGTAGGAACTCACGAA	CTCCACCTTTACCGCTTATCTT
7	BK-2	35557502	62	ATCTTGGTGGAGGAATATCTGT	GCATTATCTCAACTGGAGCAAT
8	BK-3	29416415	120	TGTTGTTGGTAGAGGTGGAATG	TAGAAGCATGAGGTCCTTGAAC
9	BK-4	36117951	133	TGCCTGTAACCTTGGAGGATTA	ACGGTGAGCCTGTGATACG
10	BK-5	5187679	120 & 189	GCTAGAATATCTGGTATGCCTGAA	CCTTCCTCAATCATCCTATCTACA
11	BK-6	29454595	133	GGATTGATTGCCAGCGAACT	GAGAACCTGTAATGCGTGCTAT
12	BK-7	35559600	62	GCAGTCCAATATCGAGCAGTAG	ATACGCCATAGCACTCACAAC
13	BK-8	35557603	62	TCCTGGTTCCACATCTACTAAG	TAGAAGACCTCCTCTGCCTAC
14	BK-9	32507244	133	TGCGTAACATCCGTCATTCG	GGCTATCTCATCCAGTTCATCC
15	BK-10	61797406	133	CGTCGGCGTCCATCATCAT	CGTCAGCAGAATGGCATCTATC
16	BK-11	29609155	133	TTGAACCAGCGTGCCATTG	AGGTATATCCGTAGTGCCAAGG
17	BK-12	35557787	62	AGGCGAAGGCGGAATAGTAG	TCCTCACGAAGAACACGAAGT
18	BK-13	24253728	120 & 189	ATAGTTGGAAGAGGCGGACTT	ATGAGCACCTACAGATGATCCT
19	BK-14	19962694	120 & 189	AATAGTTGGAAGAGGCGGAATG	AATCACCTACTGGCACTGTTC
20	BK-15	5302286	133	GGATGGAGCACGCCTGTAA	CGATACCGCCTTACTGTTCAAG
21	BK-16	30665743	120 & 189	GCTAGATTGTCAGGTGTTCCAG	AGCGTCAACATTACCGTTAAGT
22	BK-17	32506522	133	ATAGGCGGTGCTGCTGTT	CCAATGCTTCCAATTCGTCTTC
23	BK-18	30665218	120 & 189	GGCAGTGGCTAAGAGGTATG	GCCTTCTCCATCAAGAGCATTA
24	BK-19	30661099	120 & 189	GACCTCACGCTTCTAACCTTG	GCTACATCCGCTAACTCATCTG
25	BK-20	30618188	133	CTCCGTTGCTCGTCCGTAA	CGCTACATCTTCCAGTTCATCC

Table 2 The effects of TCS on hydrolysis, acidogenesis, acetogenesis and methanogenesis processes.

Time (d)	TCS (mg/L)	Hydrolysis		Acidogenesis		Acetogenesis		Methanogenesis	
		BSA degradation (%)	Dextran degradation (%)	Amino acid degradation (%)	Glucose degradation (%)	Butyric acid content (mg/L)	Acetic acid content (mg/L)	Acetic acid content (mg/L)	Methane production (mL)
1	0	55.0±1.5	71.9±1.5	87.2±0.9	74.3±1.5	7143±320	17.5±4.2	6824±222	96.2±10.3
	2.2	56.3±1.0	74.1±1.4	90.1±0.7	88.8±1.4	7106±293	5.2±0.9	6883±178	89.7±11.1
	11	57.0±1.9	70.9±2.3	83.8±1.1	44.7±2.6	7454±205	10.8±1.3	7002±291	62.4±9.7
2	0	60.2±0.7	77.4±1.1	89.2±0.8	78.7±1.9	6767±286	132.5±9.4	6436±240	149.1±12.7
	2.2	61.7±0.7	78.5±0.9	93.3±0.7	91.7±1.1	6984±240	100.7±5.5	6466±259	143.6±15.6
	11	57.7±1.2	75.7±1.0	85.9±0.7	55.9±2.0	6996±175	20.1±2.6	6581±140	103.5±12.3
3	0	64.2±0.5	85.4±1.5	90.5±0.4	88.4±1.2	6241±311	198.5±12.8	5497±197	186.2±14.2
	2.2	62.3±0.6	83.3±1.7	95.4±0.5	94.6±1.2	5549±299	161.8±11.6	5355±302	194.9±18.1
	11	60.7±1.0	81.9±1.2	88.6±0.9	69.2±2.0	6481±201	60.2±5.2	5666±188	135.8±10.8
4	0	66.9±0.9	87.2±0.9	92.2±0.3	95.0±0.9	5969±272	268.5±22.7	5404±305	244.6±13.3
	2.2	64.4±1.1	86.0±0.7	96.8±0.3	96.8±0.6	5096±188	221.9±24.4	5050±188	238.9±15.0
	11	62.3±0.7	86.6±0.8	89.6±0.7	74.1±1.8	5286±199	66.4±6.1	5139±215	177.3±14.5
5	0	68.5±0.6	88.1±1.1	93.3±0.4	97.6±0.5	5217±246	323.0±18.3	4806±149	284.1±20.4
	2.2	66.3±1.3	87.5±0.9	98.5±0.2	98.9±0.2	4547±228	262.2±31.2	4655±120	289.4±22.3
	11	67.5±0.8	87.9±0.7	91.8±0.7	77.4±2.0	4998±120	70.8±4.8	4901±300	204.6±14.7

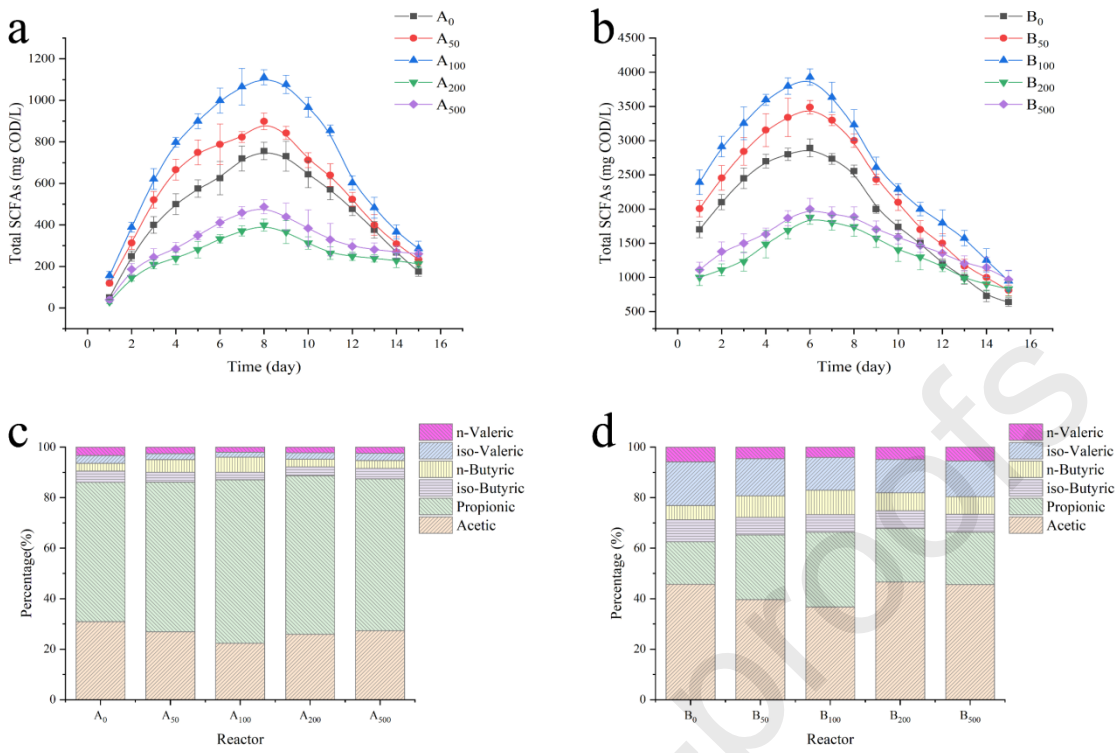


Figure 1 Different concentrations of TCS affect the accumulation of short-chain fatty acids (SCFAs). (a) Effects of different concentrations of TCS on the production of SCFAs by anaerobic fermentation of raw sludge; (b) Effects of different concentrations of TCS on the production of SCFAs by anaerobic fermentation of alkaline pretreated sludge; (c) The proportion of each composition in the Group-A to the total SCFAs on the 8th day; (d) The proportion of each composition in the Group-B to the total SCFAs on the 6th day. Error bars represent standard deviations of triplicate tests.

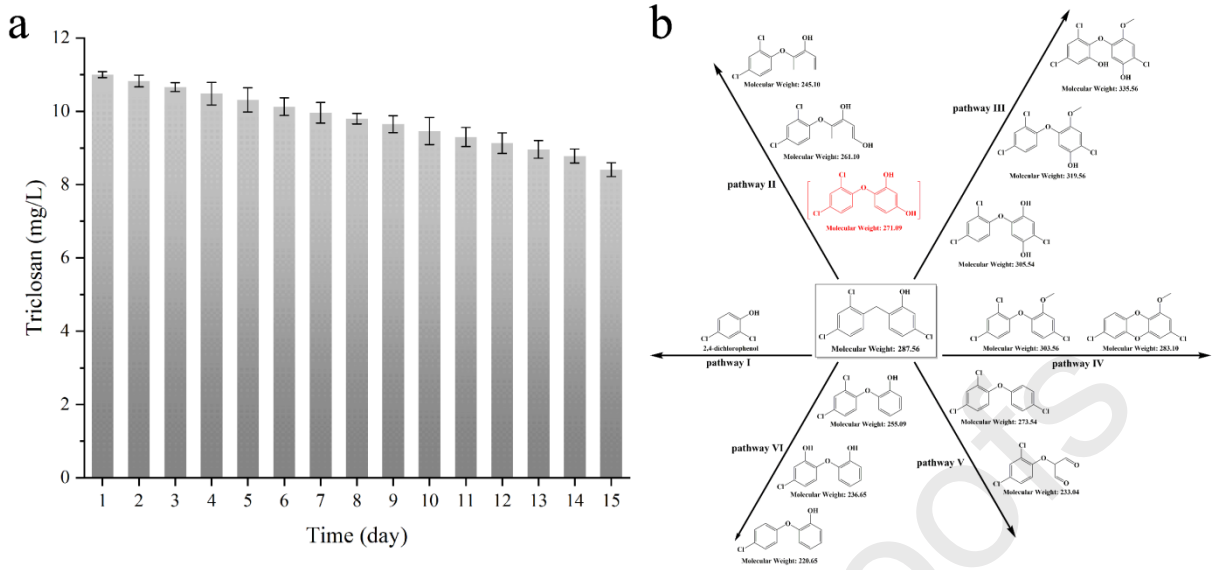


Figure 2 (a) Concentration of TCS in the A_{500} in 15 consecutive days; (b) Possible degradation pathway of TCS in the sludge anaerobic fermentation system, the product marked in red is a hypothetical intermediate.

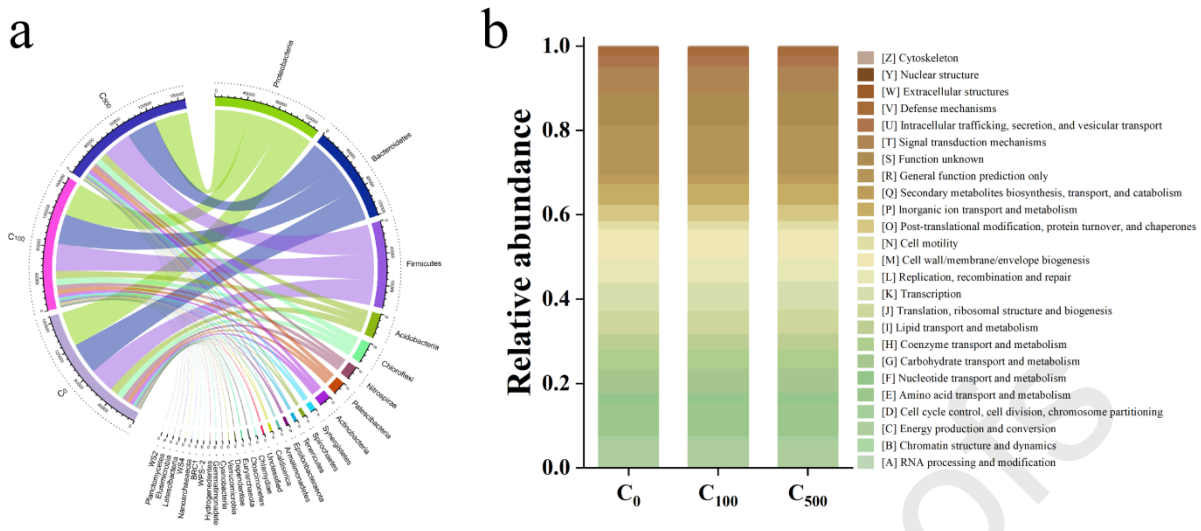


Figure 3 (a) The distribution of OTUs under the phylum-level classification in the long-term reactor; (b) Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. Results from three parallel sample determinations.

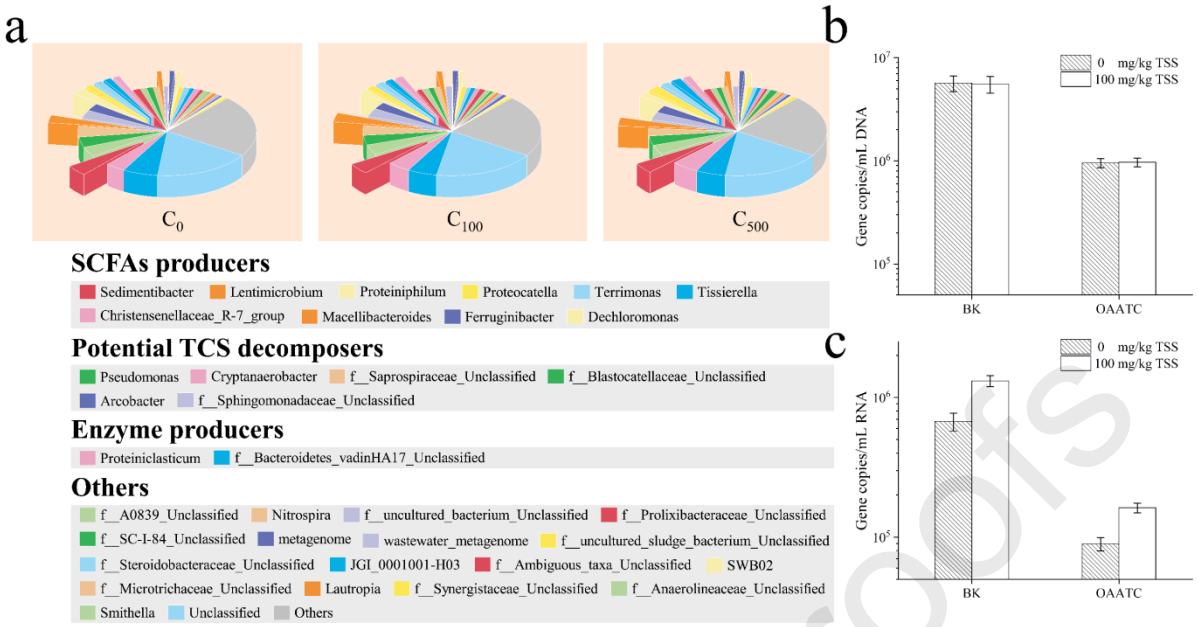


Figure 4 (a) The distribution of bacterial populations at genes level in the long-term operated reactors (b) DNA copy number of BK and OAATC in long-term sludge fermentation reactors. (c) The mRNA abundance of BK and OAATC in long-term sludge fermentation reactors. Error bars represent standard deviations of triplicate tests.

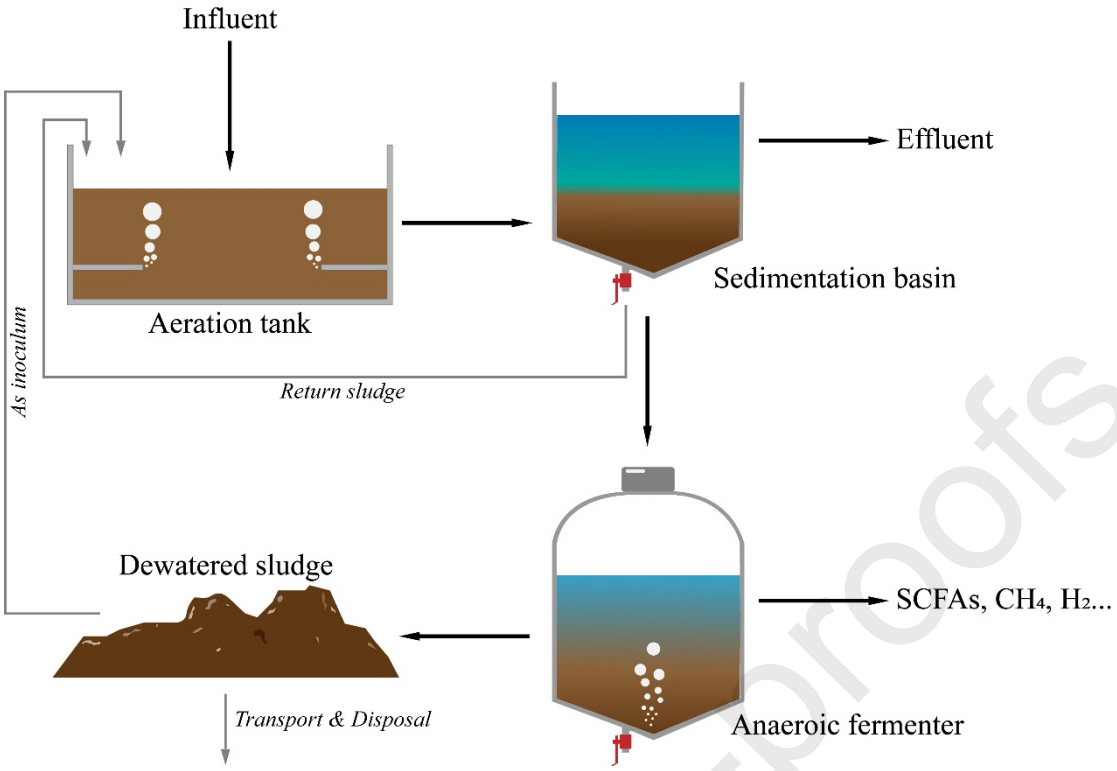
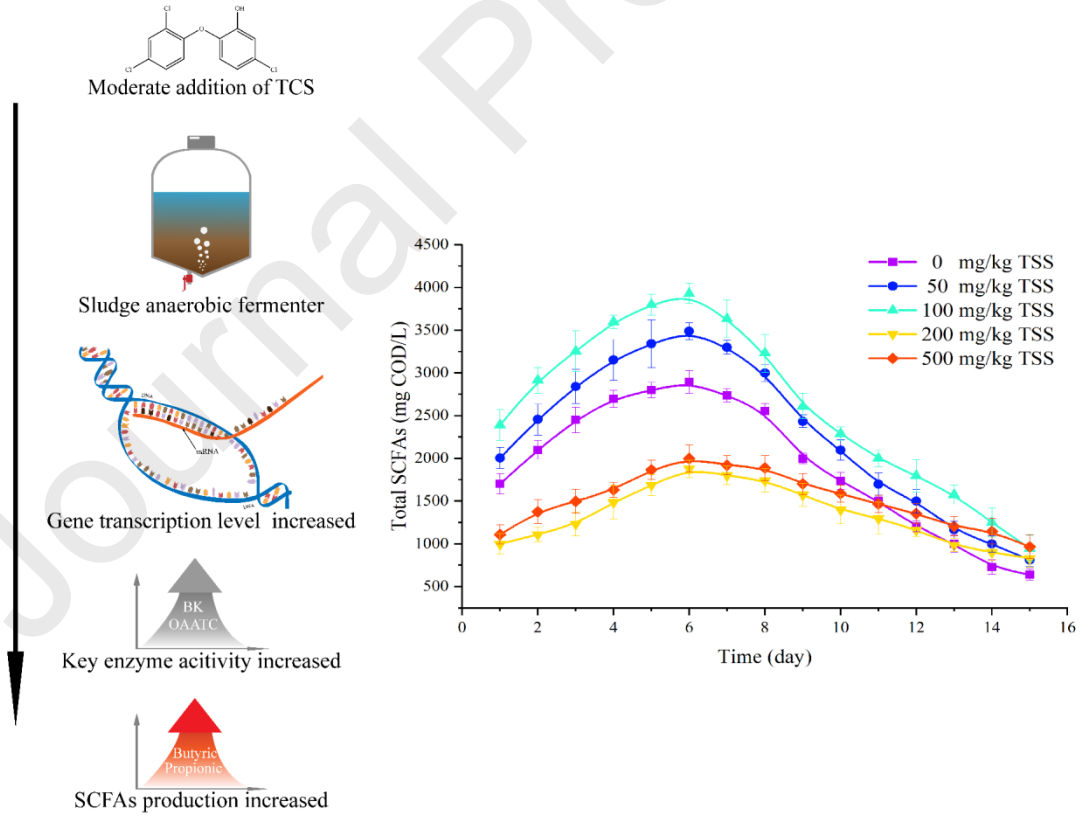


Figure 5 Schematic diagram of WWTPs.



- TCS affected SCFA production from sludge anaerobic fermentation system.
- Appropriate level TCS increased SCFA production by enhancing acidogenesis.
- High level TCS inhibited SCFA production.
- TCS didn't change the structure of anaerobic fermentation microbial community.
- Suitable TCS up-regulated the expression of acidogenesis-related enzymes.