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1 **Impact of nano zero valent iron on tetracycline degradation**
2 **and microbial community succession during anaerobic**
3 **digestion**

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16 **Abstract**

17 Supplementing nano zero valent iron (nZVI) is an attractive technology for
18 wastewater treatment due to its advantages in accelerating the hydrolysis,
19 fermentation and anaerobic digestion (AD) process. In this present study, nZVI was
20 added to investigate its effects on enhancing tetracycline (TC) wastewater anaerobic
21 treatment and the changes of microbial community, especially for underestimated
22 syntrophic-methanogenic associations. The TC concentrations were 1, 10, 30, 50, 80,
23 100 and 150 mg/L with 0.38g nZVI (with iron g/g VS of 0.50) complemented into
24 reactors. Results revealed that nZVI could enhance AD process in both control and
25 TC dosed systems, and the promoting effect on methanogenesis was more significant
26 in systems of high concentration TC, with 100 and 150 mg/L. In addition, cumulative
27 CH₄ production for all TC added systems without nZVI were higher than the control
28 indicating TC had positive effect instead of expected negative effect on AD process,
29 high TC concentration of 100 and 150 mg/L only affecting the increase factor rather
30 than causing inhibitory effect. After digestion, TC was largely removed in
31 with/without nZVI systems. And also, nZVI evidently altered the bacterial and
32 methanogenic community structure, with an increase abundance of
33 syntrophic-methanogenic associations (*Syntrophobacterales* and *Methanosarcinales*)
34 and resulting in the enhancement of methane generation. This research provides an
35 efficient method for TC wastewater anaerobic treatment.

36 **Key words:** Anaerobic digestion, Nano zero valent iron, Tetracycline, Microbial
37 community, Syntrophic-methanogenic associations.

1. Introduction

With the rapid development of industry and agriculture, incrementally large amounts of antibiotics have been applied in our society. And the antibiotics were extensively obtained much attention for their acute and chronic toxicity and side-effect to human beings and other environmental organisms. It was reported that the discharge of pharmaceutical wastewater has been considered as one of the most important sources of antibiotics in surface and groundwater, which led to huge potential human health effects [1]. Especially for tetracycline (TC), its excellent inhibitory effect on different bacterium and important new use resulted in its extensively application and in turn much higher production. As the result, the concentration of TC in effluents produced by hospital and TC production industry is higher than 100 mg/L, which was much higher than concentration of TC in wastewater treatment plants (WWTPs) [2]. Therefore, the disposal of wastewater containing TC was of great importance and challenge.

Nowadays, the most main treatment methods for degradation antibiotics are physical, chemical and biological technologies. Biological treatment has turned out to be a promising and economical approach to deal with various recalcitrant pollutants [3,4]. In addition, as the high biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and wide range of pH from 1 to 11 of pharmaceutical wastewater, physical and/or chemical methods are not the suitable

choice for their low dissolved COD removal efficiency and high consumption of chemicals [5]. Therefore, biological anaerobic method was verified as the optimal choice for its advantage in energy generation with degradation of organic matters and antibiotics or via microorganism [6]. A combination anaerobic-aerobic treatment system was also developed for removing organic matter from the high-strength pharmaceutical wastewater, and it revealed that antibiotics removal process was mainly occurred in AD phase [1]. Previous study had pointed out that AD process can effectively treat some of by-end products of pharmaceutical manufacturing wastewaters, such as TC, with TC removal rate from 14% to 89% [7]. However, anaerobic treatment is a unique process that requires sequential degradation steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) by several bacterial and archaeal groups. While, antimicrobials may directly inhibit biological activities of these microorganisms even deteriorate and collapse anaerobic processes [8]. Lu et al. [9] found that the presence of TC (250 µg/L) had a large negative effect on CH₄ and CO₂ production, and indicated that methanogenesis process was sensitive to TC presence with no insensitivity for acidogenesis. It indicated anaerobic biological method also has drawbacks for higher TC wastewater treatment efficiency. Therefore, new technology on the basis of anaerobic biological method was required to resolve this drawback.

Recent research found that starch-nZVI (nano zero valent iron)/ZVI could remove with adsorption, degradation flocculation, oxidation and reduction [10, 11].

79 ZVI can act as reducing agent which helps create an enhanced anaerobic environment
80 to improve the performance of anaerobic process and maintain a stable and favorable
81 condition for methanogenic archaea [12, 13]. In addition, the nZVI addition presented
82 many advantages such as acting as electron donor, increase the total consumption of
83 hydrogen methanogens and activity, release Fe^{2+} into the anaerobic system and
84 participating in the synthesis of the key enzymes, provide for iron element serving as
85 trace element for methanogens [12], optimizing the structure of microbial population,
86 changing the hydrolysis fermentation types and promoting the acetic acid content [12,
87 14]. Based on above, supplementing nZVI into anaerobic TC wastewater treatment
88 system might be an effective technique for both TC removal and organic matters
89 degradation. And the variations of relative abundance of microbial communities
90 affected TC removal and organic matters degradation [5], especially for changes of
91 syntrophic bacteria which are closely related to methanogens and determined the
92 efficiency of AD, are not currently well understood in these systems. It had reported
93 that supplementation of ZVI has a positive influence on the syntrophic microorganism
94 in an organic fraction of municipal solid waste AD reactor, and the syntrophic
95 interaction of acetogens and methanogens is critical to the performance of anaerobic
96 antibiotics (includes TC) treating reactors [15]. In addition, the relationship between
97 TC concentration and anaerobic performance in nZVI as additive systems, which
98 related to the tolerable concentration when apply this technology in practice, is still
99 unclear. As such, comprehensive research into this system is required to evaluate the

performance of AD process, TC degradation and variation of microbial structure, focusing on the relationship between syntrophic associations' succession and experimental conditions.

Therefore, the objectives of this research were two aspects: i) to develop an anaerobic technology for treating TC wastewater efficiently by adding nZVI and investigate the promoting effect of nZVI on AD process and TC removal in different TC dosing reactors; ii) to understand how the nZVI and TC change the structure and relative abundance of microbial communities especially for syntrophic-methanogenic associations. Therefore, nZVI was served as an additive and supplemented into anaerobic treatment systems with different concentrations of TC. The pH values, ORP values, and COD removal rates and TC removal rates were determined for evaluating the operation of anaerobic systems. And also, the methane production in each reactor was calculated. Illumina MiSeq sequencing was operated to characterize the microorganism succession and changes in microbial diversity. This research can give guidance for TC wastewater treatment by anaerobic biological technology. And also, functional anaerobic microbial communities obtained in this study can provide data support for deeply understanding of anaerobic microbial metabolism.

2. Materials & Methods

2.1 Chemicals and stock solution

Iron powder (99.9% metals basis, 100 nm) was obtained from Sigma chemicals. The

stock solution of TC (Sigma) was prepared with the concentration of 2 g/L. Substrates were supplied by glucose (99.8%, Sinopharm Chemical Reagent Co., Ltd), and stock solution was prepared with 10 g/L. In addition, H₂O was prepared for consuming oxygen.

2.2 Inoculum and Medium

It had reported that granular sludge had higher tolerance to antimicrobials than disrupted sludge [16]. Therefore, granular sludge, obtained from a mesophilic up-flow anaerobic sludge blanket (UASB) digester (pH=7.4 ± 0.2) for critic-acid wastewater AD in Green Environmental Technology Company, Xuzhou, was served as inoculum in this study. The average diameter of studied granules is about 1.5 ± 0.3 mm. The volatile solid (VS) and total solid (TS) were 51.08 g/L and 60.80 g/L, measured referring to standard method [17]. The basic anaerobic medium was prepared as described in previous study [18].

2.3 Operation of anaerobic batch reactor systems

Experimental set-up for anaerobic biodegradation was revised from Angelidaki and Sanders (2004) [18]. The batch experiments were performed in 310 ± 1 mL serum bottles in triplicate with working volume of 100 mL. 15 mL granular sludge, 25 mL basic anaerobic medium (pH= 7.0), 50 mL glucose stock solution (synthesis of TC wastewater with final concentration of 5000 mg COD/L in batch reactors and F/M of 0.65 g COD/g VS), 1 mL Na₂S·9H₂O (2.5%) distilled water were added to serum

bottles, and the rest of 9 mL consisted of TC solution and distilled water. In order to take both low concentration of TC, like in municipal wastewater and high concentration of TC in pharmaceutical wastewater into consideration, two sets of batch experiments were conducted, with the TC concentration of 0, 1, 10, 30, 50, 80 mg/L and 0, 100, 150 mg/L, which stand for the concentration of TC in WWTPs and antibiotics producing industries effluents, and finally, the reactors were flushed with 99.99% N₂ for 15 minutes. Additionally, in order to reveal the impact of nZVI on anaerobic system, 0.38 g iron powder was complemented into half of experimental reactors, with the ratio of iron g/g VS of 0.50, which was proved as optimized value in previous study [19]. For maintaining anaerobic condition, serum bottles was flushed with 99.99% N₂ for 15 minutes and then sealed with rubber stoppers and aluminum caps. Batch reactors were incubated in a thermostatic shaker at 37 ± 0.5 °C and 160 rpm. During the incubation, gas production was monitored every day.

2.4 Analytical methods

The analysis of methane content was performed via gas chromatography (FULI GC9790II) with column of TDX-01 (2m long and 3mm in inner diameter). Argon serves as carrier gas. The temperature of packed column, detector and injection port is set to 120 °C, 160 °C and 160 °C respectively.

The cumulative volume of CH₄ generated in serum bottle is calculated by multiplying the headspace volume (260 mL) by the CH₄ percentage (mLCH₄/mL) in

the headspace as determined by GC analysis. It must be noted that gas samples taken from batch reactors should equilibrate in batch reactors which make the pressure in batch reactors have been taken into consideration. In addition, the obtained value of cumulative CH₄ production was normalized in Standard Temperature and Pressure (STP) conditions (0 °C and 1 atm) according to ideal gas law ($PV = nRT$). The methane production assay is referenced to sample mass or chemical oxygen demand (NmLCH₄/g VS or NmLCH₄/g COD) [18].

COD, pH value and reduction-oxidation potential (ORP) were determined according to Standard Methods [17]. The concentrations of TC in liquid and sludge samples were measured by LC/MS (ABI 6500), the detailed protocol referred to Cetecioglu et al. [20].

2.5 DNA extraction and Illumina MiSeq sequencing process

Sludge samples obtained from batch reactors at the end of AD. DNA from sludge samples of each reactor was extracted from 0.5 g of sludge, after centrifuging of 50 mL sludge for 10min at 12000 rpm, using Fast DNA Spin Kit for Soil (MP bio, USA). Detailed procedure of DNA extraction was followed the manufacturer's instruction. The DNA concentrations were determined by Nano drop 1000 (Thermo Scientific).

PCR amplifications for bacteria and archaea were conducted using the primers sets 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3') for the V3-V4 region of 16S rRNA gene [21]

and 524F10extF (5'-TGYCAGCCGCCGCGGTAA-3') and Arch958RmodR (5'-YCCGGCGTTGAVTCCAATT-3') for the V4-V5 region of 16S rRNA gene [22], separately. The mixtures of the amplicons from two sets of primers were used for sequencing on Illumina MiSeq platform following standard protocols, which were performed in Major bio China.

2.6 Modified Gompertz model

Recently, many studies have used the modified Gompertz model to predict biogas (CH₄ and CO₂) production potential by assuming that the rate of gas production is proportional to the microbial activity in anaerobic digesters [23, 24]. The modified Gompertz equation is shown as follows:

$$P = A \times \exp \left\{ -\exp \left[\frac{Ue}{A} (\lambda - t) + 1 \right] \right\}$$

Where

P - cumulative methane production, N mL/g VS at any digestion time t;

A - methane yield potential, N mL/g VS;

U - maximum rate of methane production, N mL/ (gVS•d);

λ - lag phase period to produce methane, days;

t - digestion time at which cumulative methane production P is measured, d;

e - mathematical constant (2.718282).

The kinetic parameters of A, U, and λ were simulated for each batch reactor using non-linear regression with the help of SPSS software. From the above equation,

kinetic constant of CH₄ production rate will be expressed by U constant.

2.7 Statistical analysis

All assays were performed in triplicate. Data analysis was done with EXCEL 2017. Additionally, Origin 8.0 was applied for production of graphics. To compare the differences in microbial communities between nZVI added reactors, TC dosed reactors and blank, a one-way analysis of variance (ANOVA) was used. Analyses were conducted on the free online platform of Majorbio I-Sanger Cloud Platform (www.i-sanger.com). A *p* value less than 5% was considered to be statistically significant.

3. Results and discussion

3.1 Performances in pH, ORP and COD removal

As taking liquid sample during the process of digestion operation can significant impact the CH₄ production and substrate consumption in batch reactors with work volume of 100 mL, the pH and ORP values were only measured at the end of digestion to evaluate the final environmental condition of each system. The pH values in batch reactors with nZVI addition were significant higher than those without adding nZVI systems. It indicated that adding nZVI could release the volatile fatty acids (VFAs) accumulation, which is beneficial to AD process [25]. For batch reactors with TC concentration range of 0 - 80 mg/L, the pH variation between reactors with

and without nZVI addition was slight, while it was significantly varied with TC concentration of 100 and 150 mg/L. It means that nZVI supplementation had significant positive effect on AD process in the systems with high TC concentration. These results coincided with the COD removal rates in these reactors, nZVI added systems presented higher COD removal rates than those without nZVI systems at each level of TC concentration. Similarly, the large difference between with and without nZVI systems was occurred under high TC concentration of 100 and 150 mg/L. The nZVI dosed reactors maintained the pH at the level of 7.3 - 8.3, which might be due to that the nZVI served as an acid buffer by the dissociation of Fe^0 and Fe^{3+} (reaction 1, 2, 3) [26, 27].

In addition, the low pH values (5.2-5.5) occurred in blank 2 system and systems with TC concentration of 100 and 150 mg/L, and their COD removal rates and cumulative methane yields were also low. These could be explained by that the low pH condition influenced the growth of methanogens.

Coincidentally, the ORP values in nZVI dosed reactors were higher than those in without nZVI added systems. However, the result was contrary to previous outcomes that nZVI could lower the ORP value to maintain a reductive condition for methanogenic archaea [14]. Nevertheless, the ORP values in nZVI added systems

were -314 ~ -261 mV, which still meet the anaerobic requirement for methanogenesis (ORP < -200 mV). Among the nZVI supplemented with different TC concentration systems, the ORP values from mixed liquid slurry ranged from -261 to -296 mV at the end of AD, suggesting that all the dissolved iron species was in the ferrous form (e.g., Fe^{2+} or $\text{Fe}(\text{OH})^+$), which may lead to its adsorption and precipitate (e.g., for forming FeS) in AD [27]. The increased ORP value also might have relationship with fermentation type occurred in nZVI systems. And, considering the pH values, the fermentation type in nZVI added systems might be butyrate fermentation with ORP of -350 ~ -200 mV and pH value > 5.8. Although the concentration of butyrate had been not determined in batch reactors because of the high COD removal rate, we could deduce that butyrate was main intermediates in nZVI added systems owing to the increase abundance of *Syntrophus* containing groups for syntrophic butyrate-oxidizing (Fig. 3).

It was obvious that COD removal rates in blank 1 and non-nZVI systems with TC concentration of 1 - 80 mg/L were quite similar to those with nZVI systems, with the values approximately of 92% - 96%. This also illustrated that adding nZVI had little impact on COD removal rate under low TC concentration (0 - 80 mg/L), which might due to the COD removal rates in non-nZVI reactors had reached a relative high level. Interesting, TC addition (0 - 80 mg/L) had not showed evidently negative effect on COD removal rate, this result was corresponded to methane production in these reactors. The COD removal rate in blank 2 was quite lower than that in blank 1, which

means that activity of anaerobic bacteria in blank 2 was much lower than that in blank 1. But the high removal rate of COD in reactor of blank 2 with nZVI revealed that nZVI could enhance AD and active anaerobic microbes. The similar result occurred in 100 and 150 mg/L TC added systems, COD removal rates were only 50.04% and 49.18%, respectively, while in these two levels of TC with nZVI added systems, the COD removal rates reached as high as 94.80% and 94.18%. These results indicated that nZVI could enhance AD process in systems containing high concentration of TC. As mentioned above, this might due to that nZVI can be used as electron donor, accelerating the hydrolysis and fermentation process and increase the methanogenic activity, release Fe^{2+} into the anaerobic system and participate in the synthesis of the key enzymes [12].

3.2 The methane production kinetics and TC removal

To deeply investigate the effect of adding nZVI on AD process and TC degradation performance, the cumulative methane production and TC removal efficiency were tested and the methane yields were also listed in Table 1. Compared with the non-added nZVI systems at same TC concentration level, adding nZVI could obviously enhance the methane production. It was also verified by the change of increase factors that higher TC concentration gives higher increase factor. Meanwhile, an interesting result can be observed that CH_4 production in batch reactors with TC have higher cumulative methane yield than that of blank reactors, which means that

TC has positive effect on methane production. And this result was contrary to some previous studies. In fact, TC can inhibit the AD performance via influencing the metabolism, growth and viability of various microorganisms. Previous studies delivered different results due to differences in the setup (batch reactor vs continuous process), substrate utilization (e.g. manure, activated sludge, biogas plant fermenter content), initial compound concentration and duration of the experiment. Some researchers also reported that TC showed no relevant change in methane generation compared to the control at exposed dose of 10 mg/L, furthermore, it also stated that TC had no significant effects on AD performance [16], whereas other studies revealed a decrease of the biogas and/or methane yield [20]. Shi et al., [28] found biogas production decreased by 25% at TC dose of 50 mg/L. The COD removal rates between blank and experimental reactors with TC concentration lower than 80 mg/L were quite similar (seeing Table 1 again), but the increased CH₄ production in TC added systems than blank means that TC enhanced the proportion of methane in biogas, and this result might cause by the microbes variation (discussed in 3.3).

Owing to the fact that the TC could be applied to generate biogas during AD process, the specific methane production was calculated and the data were simulated by utilizing modified Gompertz model. The predicted curves for specific CH₄ yield along with digestion time fit well with observed values in each system, with $R^2 > 0.90$ (Fig.1, Table 2). It revealed that nZVI had positive impact on methane production in both blank and TC added systems, with higher methane yield potential. These results

could be explained by the recent research outcomes that adding nZVI in AD improved methane yield due to the release of hydrogen during nZVI corrosion/oxidation, which can serve as the electron donor for methanogens [12]. On the other side, the rapid release of hydrogen during nZVI corrosion at a time would result in a high partial pressure of hydrogen, which can inhibit the methanogens to some extent at the beginning of AD process. As the anaerobic digestion proceeded, hydrogen was consumed gradually, and partial pressure of hydrogen decreased, methanogens continued to generate methane with the stimulation by nZVI. These could explain why the lag times (λ) in nZVI added systems were higher than non-nZVI systems, and it also give an reason for the lower maximum methane production rate (U) in system with nZVI than without nZVI [12]. Some reports pointed out that nZVI addition could inhibit methanogenesis for its disruption of cell integrity [29]. While, in this study, the enhancement of methane production in nZVI added systems indicated that even if cell disruption occurred in studied reactors, functional microbes involved in methanogenesis were not disrupted, and the increased soluble COD would be consumed by anaerobic microorganism to partly enhance the methane generation. In non- nZVI added systems, reactors with TC dosed showed significant higher methane yield potential values than blanks (such as 28-30 >18.85 NmLCH₄/gCOD), it indicated the positive effect of TC on CH₄ production occurred, but it was closely related to the TC concentration (Table 2). This was reasonable that high TC concentration has negative effect on the activity of anaerobic microorganism [26].

Whereas, the promoting effect of nZVI on methane production in TC wastewater treatment system was independent on TC concentration, even in high concentration of 150 mg/L with cumulative methane production and increased factor of 162.10 NmL and 4.87, respectively. Therefore, it could be concluded that nZVI has positive effect on anaerobic TC wastewater treatment, and the promoting effect was not affected by the concentration of antibiotics.

At the same time, the TC was biodegraded in studied batch reactors and the removal rate results were presented in Fig. 2, which showed that the removal rates varied from 68% to 98%. Besides, the TC degradation rates in all systems were higher than 90% except the system with TC concentration of 1 mg/L. The high TC removal rates of non-added nZVI reactors revealed that TC could be biodegraded by microorganism in studied granular system. Previous study also reported that TC could be degraded by 70% during pilot-scale composting experiment [30]. Although Cetecioglu et al. found a biodegradation rate of 46% for TC under methanogenic condition [13], Cetecioglu et al. [20] also showed the major fraction (> 80 %) of the TC introduced into the anaerobic reactor could be fully or partially biodegraded along with the organic substrate. The high removal rates of TC in studied anaerobic reactors could be resulted from the granular sludge which owned higher tolerance to TC than disrupted sludge [16]. In addition, it also might be caused by the activity of microorganism, which had a certain capacity for TC removal without relation to TC concentration. And the residue of TC mainly adsorbed by granular sludge, accounting

for more than 95% of the total remaining TC , and very little concentration of TC detected in liquid phase (Fig. 2). This result was similar to previous finding that TC showed a strong sorption to biosolids such as soil or activated sludge [31].

The effect of nZVI on degradation and adsorption of TC has been reported by Chen et al. [32]. The degradation products mainly included the 4-epi-TC and loss of amine and water as TC-like matter. The iron (hydroxyl) oxides were transformed from nZVI in solution, and then adsorb the TC. The TC is an efficient chelating agent for metal ions with high valent, which would chelate with the iron ion as Fe-TC complex in solution [33]. This could explain the higher removal rates of TC in nZVI dosed systems. Fu et al. [10] obtained 99% TC removal rate when 500 mg/L TC was processed with 0.2 g/L nZVI. In addition, during the process of acidogenesis, the pH value in reactors decreased, and the released H^+ would result in the corrosion of nZVI and producing more active hydrogen (H^0) for reducing TC with oxidizing Fe^0 to Fe^{2+} ($Fe^0 + RX + H^+ \rightarrow Fe^{2+} + RH + X^-$) [34]. The release of Fe^{2+} also supported the ORP increase in nZVI added systems as mentioned in 3.1.

As can be seen from the Fig. 2 that the nZVI enhanced the TC removal, but the improvement of TC removal rates in nZVI added systems were not obvious, with enhanced removal rates lower than 4%, expected that in system with 1mg/L TC added of 8.7%. Because the TC removal rates in non-nZVI system were relatively high, and the difference of TC removal rates between nZVI and non-nZVI added systems was slight. It indicated that TC could be efficiently biodegraded in studied granular

systems. Therefore, we can conclude that the addition of nZVI is an effective technique for both TC removal and organic matters degradation.

3.3 Succession of microbial community structures

The relative abundance of bacteria and archaea phylum was calculated based on OTUs. Fig.3 showed the succession of the dominant bacteria order (genus) and archaea order (genus) (relative abundance >2%) of the microbial communities, respectively.

3.3.1 Effect of TC concentration on diversity and abundance of bacteria and archaea

Diversity and abundance of Bacteria: Fig. 3a and Fig.3b showed the bacterial diversity and abundance in original granular sludge and granules in experimental reactors on order and genus level. The microbial structure of the two originals was quite similar because of the same source of original granules with only different sampling time. While, big difference of microbial diversity and abundance occurred between blank1 and blank 2. Compared to original 1, *Atribacteria* instead of *Bacteroidetes* served as dominant bacteria in blank 1 system, which indicated that microbial structure shifted with incubation. In blank 2, *Clostridiales* turned to be the predominant bacteria. The variation between blank 1 and blank 2 could explain the big variation of methane production and COD removal rates between these two systems.

On the order level, compared to the original sludge, reactors without nZVI had a higher abundance of *Atribacteria*, and batch reactors with nZVI had higher abundance of *Anaerolineales* and *Syntrophobacterales*. The *Syntrophobacter* is important functional bacteria in the process of anaerobic methanogenesis from organic matters, oxidizing the aromatic compounds incompletely to acetate. And it can only use a few kinds of substrates such as propionate and butyrate in co-culture with H₂-scavenging partners. Organisms of this group, like *S. aciditrophicus* and *Syntrophus buswellii*, are syntrophic butyrate degraders, and several bacteria strains, (such as *S. fumaroxidans*, *S. wolinii*, *S. pfennigii*, *S. sulfatireducens*, *P. thermopropionicum*, *P. schinkii*, *P. propionicum*, *Smithella propionica* and *Desulfotomaculum thermobenzoicum* ssp. *Thermosyntrophicum*) belonged to *Syntrophobacter* are propionate-oxidizing bacteria [35]. The abundance of *Syntrophobacterales* in TC added systems with low concentration (1-80 mg/L) and high concentration (100 mg/L and 150 mg/L) were higher than those in blank 1 and blank 2, which means that the increase abundance of this bacteria could enhance AD process to finally promote methane production. This could give an explanation for the higher methane production in nZVI added systems, since the abundance of *Syntrophobacterales* had positive correlation with methane production. It was also verified by the results that the higher abundance *Syntrophobacterales* led to higher methane production, including TC1 to TC80 and nZVI added systems, lower abundance *Syntrophobacterales* led to lower methane production, including blank 2, 100 mg/L and 150 mg/L TC added systems. Therefore,

we could deduce that methane production enhanced by TC addition and nZVI was partly owing to the increasing abundance of *Syntrophobacterales*. This result is in agreement with the observations made by Kong et al. [15], who reported that supplementation of ZVI has a positive effect on the syntrophic microorganism in AD reactor treating solid waste. In addition, Meng et al. [36] found that ZVI addition into an anaerobic reactor increased the propionate degradation rate compared to the control.

On the genus level, we can observe that nZVI added systems had quite similar microbial structure, which means that nZVI could change the microbial diversity and abundance independent on the concentration of TC. *Syntrophohabdus* significantly increased in all nZVI added systems compared to original and non-nZVI systems. Therefore, syntrophic bacteria could be served as an important index for evaluating the performance of AD.

Diversity and abundance of Archaea: In original system, *Methanobacteriales* was predominant methanogens with proportion of 86%, and *Methanosarcinales* also occupied a certain proportion about 7%, while *Methanomicrobiales* only owned taken up lower than 1%. After incubation, the structure of archaea clearly changed. In the first set experiment (contains blank 1, TC concentration from 1 to 80 mg/L), the relative abundance of *Methanosarcinales* increased and turned to be the predominant methanogens with proportion of 63.55% - 77.02% in these systems. While in second experimental set (including blank 2, TC concentration of 100 and 150mg/L), the

abundance of both *Methanosarcinales* and *Methanomicrobiales* raised, and *Methanobacteriales* was still the predominant methanogens (Fig 3c and Fig. 3d). What was interesting, the archaea structure and abundance in blank2 with nZVI added and 100 mg/L and 150 mg/L TC systems with nZVI were quite similar to those in blank 1 and TC (1-80). Cetecioglu et al., [20] found that methanogens and archaea were not affected by TC in an anaerobic reactor after 120 days incubation with TC concentration of 90 ppm. It means nZVI addition could shift microbial structure and abundance, which has nothing to do with the concentration of TC. This finding could give an explanation for the similar methane production in different concentration of TC added systems.

In fact, there is an intrinsic link between the communities' structure between bacteria and archaea. As showed in Fig.3c, *Methanobacteriales*, instead of *Methanosarcinales*, dominated in systems of original_1&2, TC100, TC150 and Blank2, where *Anaerolineales* showed relative lower percentages than other systems (Fig.3a). Since acetate producer *Anaerolineaceae* and acetoclastic methanogen *Methanosaeta* are syntrophs in the acetoclastic pathway [37]. In addition, the high relative abundance of *Methanosarcinales* in systems of TC1-TC80 and Blank1 might result from the high abundance of *Aribacteria*, another acetate producer [38].

3.3.2 Effect of nZVI on microbial structure and syntrophic association

Fig. 4 described the effect of nZVI addition on the shift of microbial structure on genus level (including bacteria and archaea). It was observed that the

supplementation of nZVI in AD system resulted in a significant decrease of *Atribacteria* and increase of *Anaerolineaceae*. In addition, the abundance of *Syntrophorhabdus*, *Syntrophobacter* and *Syntrophaceae*, which plays an important role in the degradation of VFAs (butyrate and propionate) and providing sufficient substrates for methanogens during AD process, all had significant increase in nZVI dosed systems. This result provided an explanation for the enhancement of methane production in nZVI added system. In addition, the proportion of *Methanosaeta* in nZVI added system increased while *Methanobacterium* decreased in nZVI added systems. Previous study found that nZVI addition could inhibit hydrogenotrophic methanogenesis due to the rapid hydrogen production during the dissolution of nZVI [39]. This result is in agreement with the observation of Zhang et al. [5], who reported that *Methanosaeta* abundance increased from 33.81% to 57.33%, while the abundances of *Methanobacterium* reduced from 26.98% to 15.31% after GAC/nZVI addition. *Methanosaeta* was strict anaerobe and could only use acetic acid as the substrate to produce methane, while *Methanobacterium* was hydrogen nutritional methanogens which could produce methane by reducing CO₂ with H₂ and formic acid. In nZVI added systems, the abundance of *Syntrophus* (typical hydrogen-producing acetogenic bacteria using butyric acid) increased (mentioned above), resulting in accumulation of acetic acid, which would promote the growth of *Methanosaeta* and then consumed more acetic acid. It was known that *Methanosaeta* was also positively correlated with methane production.

One-way ANOVA analysis was conducted between original sludge, blank system without TC and nZVI, blank system with nZVI, systems with TC addition and systems with both TC and nZVI addition (Fig. 5). The results revealed that, compared to TC added systems, the proportion of *Anaerolineaceae*, *Gaiellales*, *Chloroflexi* and syntrophic bacteria including *Syntrophorhabdus* and *Syntrophaceae* has significant higher abundance than these bacteria in non-TC added system (P -value <0.05). In addition, *Methanosaeta* also has a higher abundance in nZVI and TC added systems than only TC added systems. The increasing abundance of syntrophic-methanogenic associations might be a reason for that nZVI enhanced methane production in TC added systems. This result is in agreement with the observations made by Kong et al. [15], who reported that supplementation of ZVI has a positive effect on the syntrophic microorganism in an organic fraction of municipal solid waste AD reactor, and syntrophic association among the microbial genera of *Syntrophomonas*, *C. butyricum* and *Methanosarcinales* in ZVI dosed systems plays a significant role both in acidification elimination and CH_4 production promotion.

Combining the communities' structure analysis of bacteria and archaea, we found that nZVI addition could change the microbial community's structure, and the increasing abundance of syntrophic-methanogenic associations (*Syntrophobacterales* and *Methanosarcinales*) contributed to high methane production. Previous study used to focus on the change of dominant groups [5], and ignored the key microbial with a relative low abundance but playing an important role in AD process, such as

syntrophic fatty acids oxidizing bacteria (SFOB). SFOB constitute a 'keystone' guild, i.e., organisms whose impact on community structure and function is far greater than what their abundance would suggest [40]. Meanwhile, *Syntrophobacterales* plays an important role in AD process, but it is still not valued in the study focusing on wastewater degradation. Thus, it is necessary to deepen the understanding of its functions and further establish engineering control strategies in the future.

3.3.3 Effect of TC on microbial structure

As mentioned above, TC addition also had positive effect on methane production. Compared with the blank systems, the proportion of *Anaerolineaceae*, *Chloroflexi*, *Syntrophorhabdus* and *Syntrophaceae* had significant increase (P -value <0.05) (Fig. 5). Meanwhile, the abundance of *Methanosaeta* in TC added systems was higher than it in blank system, while *Methanobacterium* presented a lower level in TC added system than in blank systems. As discussed above, these shift of bacteria and archaea contributes to the promotion of AD process in TC added systems.

In conclusion, both nZVI and TC addition have significant effect on microbial community succession, in turn, influencing AD performance, including COD removal and methane generation. Therefore, the enhancement of methane production by nZVI and TC might due to the change of microbial structure with increasing the abundance of functional microorganism, like syntrophic association, during AD process, which provides a biological basis and technological supports for enhancing the treatment efficiency from TC wastewater in AD process.

4. Conclusions

The addition of nZVI is an effective technology to improve TC degradation and methane production in anaerobic process. TC removal rates reached over 90% in most systems except the reactors with TC concentration of 1 mg/L. Interestingly, TC also had positive effect instead of expected negative effect on AD, even with high TC concentration of 100 and 150 mg/L. The high dosage of TC addition just affected the increase factors methanogenesis rather than causing inhibition. nZVI evidently altered the bacterial and methanogenic community structure, with an increase abundance of syntrophic-methanogenic associations (*Syntrophobacterales* and *Methanosarcinales*) and resulting in the promotion of methane production. The results would provide the theoretical basis and technological supports for enhancing the treatment efficiency and energy recovery from TC wastewater in AD process.

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