Mechanistic insights into the effect of poly ferric sulfate on anaerobic digestion of waste activated sludge

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Abstract

Poly ferric sulfate (PFS), one of the typical inorganic flocculants widely used in wastewater management and waste activated sludge (WAS) dewatering, could be accumulated in WAS and inevitably entered in anaerobic digestion system at high levels. However, knowledge about its impact on methane production is virtually absent. This study therefore aims to fill this gap and provide insights into the mechanisms involved through both batch and long-term tests using either real WAS or synthetic wastewaters as the digestion substrates. Experimental results showed that the maximum methane potential and production rate of WAS was respectively retarded by 39.0% and 66.4%, whereas the lag phase was extended by 237.0% at PFS of 40 g per kg of total solids. Mechanism explorations exhibited that PFS induced the physical enmeshment and disrupted the enzyme activity involved in anaerobic digestion, resulting in an inhibitory state of the bioprocess of hydrolysis, acidogenesis, and methanogenesis. Furthermore, PFS's inhibition to hydrogenotrophic methanogenesis was much severer than that to acetotrophic methanogenesis, which could be supported by the elevated abundances of *Methanosaeta* sp and the dropped abundances of *Methanobacterium* sp in PFS-present digester, and probably due to the severe mass transfer resistance of hydrogen between the syntrophic bacteria and methanogens, as well as the higher hydrogen appetency of PFS-induced sulfate reducing bacteria. Among the derivatives of PFS, "multinucleate and multichain-hydroxyl polymers" and sulfate were unveiled to be the major contributors to the decreased methane potential, while the "multinucleate and multichain-hydroxyl polymers" were identified to be the chief buster to the slowed methane-producing rate and the extended lag time.

Keywords: Poly ferric sulfate; Waste activated sludge; Anaerobic digestion; Methane production; Hydroxyl polymers

1. Introduction

Poly ferric sulfate (PFS), the polymerized iron salts, can provide a variety of nucleic hydroxyl high-valence complex ions and hydrolyze to the highly cross-linked hydrophobic multinucleate and multichain-hydroxyl polymers after dissolving in aqueous phase, making it possess the high coagulation efficiency (Jiang et al., 1998; Zouboulis et al., 2008). Compared with the conventionally inorganic coagulants such as ferric chloride and aluminum sulfate, the PFS owns some advantages, such as comparatively lower dose, wider range of pH and temperature but more effective coagulation properties, and contains no harmful substances (Jiang et al., 1998).

In sewage treatment system, the potential pathway of PFS entering in sewage sludge is the wastewater pretreatment process, such as the enhanced coagulation and chemical phosphorus removal process (Fig. S1). In these processes, PFS would firstly hydrolyze to macromolecular iron-polymer containing hydroxyl and sulfate groups, and then complex and precipitate with phosphorus or sludge flocs, in which most of the PFS is inevitably absorbed and concentrated in primary sedimentation sludge (PS) (Chu et al., 2018; Moussas et al., 2009). It was reported that the dosage of PFS in wastewater pretreatment process was generally at 10-60 mg/L and highly depended on the source of wastewater quality (Chu et al., 2018; Zouboulis et al., 2008), with the potential content of Fe remained in chemically enhanced primary sedimentation sludge being in the range of 0 to 22 g per kg of total solid sludge (Ghyoot et al., 1997; Lin et al., 2017; Zhou et al., 2020). Moreover, in some small-scale wastewater treatment plants (WWTPs) where anaerobic digestion of waste activated sludge (WAS) in-situ in WWTPs is not economically feasible and in some developing countries like China where most of the WWTPs have not already been configured with anaerobic digesters, WAS is firstly required to be dewatered and then gathered together for further treatment (Fig. S1). As PFS is substantially added into WAS during mechanically dewatering process, PFS levels in such sludges are inevitably at high levels.

It was reported that PFS content in dewatered sludge was in the range of 10-40 g/kg dry sludge when the PFS was applied in sludge conditioning (Bratby et al 2016; Watanabe et al., 1999; Wei et al., 2018a). In addition, according to our survey on 4 WWTPs in Central China, 90% of the PFS used in mechanical dewatering was remained in dewatered sludge, and the PFS levels in such sludge reached at 3.0-48.5 g/kg TS. Till now, however, most of the studies on PFS focused on its performance optimization, few considered its accumulation in sewage sludge (include PS and WAS) and potential impacts on subsequent treatments, such as anaerobic digestion (Fig. S1).

Anaerobic digestion is not only a conventional technology for stabling sewage sludge, but also a developing technology to be regained in energy self-sufficient operation (Appels et al., 2008). Through anaerobic digestion, the sludge volume can be effectively reduced, the pathogenic microorganisms can be effectively killed, and more importantly, the carbon substrates can be transferred to renewable energy, methane (Appels et al., 2008; Zhang et al., 2020). It is known that sludge anaerobic digestion includes several biological conversions (e.g., hydrolysis, acidogenesis, and methanogenesis) executed by a series of microbes such as hydrolytic microorganisms, acid-producing microorganisms, and methanogens (Wu et al., 2020; Zhen et al., 2017). Thus, the accumulated PFS in sewage sludge might affect these bio-conversions, thereby affecting the performances of sludge anaerobic digestion.

When PFS is dissolved in hydrous media such as sewage sludge, sulfate, iron, and their "hydroxyl polymers" will co-exist. As a compound being rich in iron, PFS would provide the required iron element for the synthesis of iron-containing enzymes involved in hydrogenotrophic methanogenesis or aceticlastic methanogenesis, and meanwhile served as a scavenger for S^{2-} in sludge, which might be beneficial for methane production (Wei et al., 2018b). As a substance containing sulfate, however, PFS might serve as a special form of anaerobic respiration and terminal electron-accepting process, and thereby induced the

enrichment of sulfate-reducing bacteria (SRB) and competition and/or coexistence with methanogens for carbon and electrons, as well as the production of hydrogen sulfide, which could result in a shift in the metabolic pathways of organic substrates and the change of microbial community structure in anaerobic digestion (Cetecioglu et al., 2019; Hansen et al., 1999; Ozuolmez et al., 2015; Qiao et al., 2016). And as the "hydroxyl polymers" such as $Fe_3(OH)_4^{5+}$, $Fe_5O_2(OH)_6^{5+}$, which play a core role in coagulation process and have a similar but better performance with ferric hydroxide, may induce the aggregation of sludge flocs and the alteration of subsequent anaerobic digestion performance (Moussas et al., 2009). The effect of coagulant (e.g., $FeCl_3$ and PACl) on acidogenic fermentation of PS have been clearly reported by Lin et al. (2017, 2018), which demonstrated that $FeCl_3$ dosed at 10-30 mg Fe/L sewage had little influence on sludge hydrolysis and volatile fatty acid production, whereas an obvious inhibitory effect was observed for PACl in organic hydrolysis of the PS. These excellent attempts open the box of coagulant's effect on anaerobic fermentation of PS, however, the WAS has different properties compared to PS. For instance, WAS is the sludge produced by biological process and it mainly contains biomass and extracellular polymeric substances, whereas PS is the sludge composed of settleable particulate organics removed from wastewater pretreatment processes. Up to now, however, there is no information available on the effects of PFS, the mixture or synthesis being made up of sulfate, iron and "hydroxyl polymer", on the anaerobic digestion of real WAS. And moreover, the underlying mechanisms and microbial community response to PFS during anaerobic WAS digestion have not been yet thoroughly investigated.

Thus, the main objective of this work is to reveal the effects of PFS on the anaerobic WAS digestion. Firstly, the effects of PFS at different dosages (0, 5, 10, 20, or 40 g/kg TS) on methane production during anaerobic WAS digestion was investigated. Then, the underlying mechanisms for PFS affecting the digestion were identified by assessing its effect on the solubilization of WAS, the processes of WAS

hydrolysis, acidogenesis and methanogenesis, the contributions of the intermediates decomposed from PFS to methane production, the microbial community as well. To our knowledge, this is the first work reporting the adverse effects of PFS on the anaerobic digestion of real WAS. The findings obtained will provide insights into the PFS-involved anaerobic digestion system and are supposed to make a sound contribution to mitigate PFS's negative effects in the future.

2. Materials and methods

2.1. WAS and PFS

The WAS used in this study was obtained from the secondary sedimentation tank of a WWTP with sludge retention time of 20 d in Changsha, China, where PFS was not used in wastewater treatment. The WAS was concentrated by gravity thickening at 4 ℃ for 24 h and screened with a 1 mm sieve to remove impurities before use. The main characteristics of used WAS were as follows: pH 6.8 ± 0.1, total solids (TS) 38300 ± 600 mg/L, volatile solids (VS) 24800 ± 350 mg/L, soluble chemical oxygen demand (COD) 170 ± 10 mg/L, total COD 37000 ± 420 mg/L, total carbohydrate 3900 ± 160 mg COD/L, and total protein 17800 ± 220 mg COD/L. The seed sludge was collected from an anaerobic digester fed with WAS in our lab, and its main characteristics were: pH 6.9 ± 0.1, TS 16500 ± 320 mg/L, VS 12300 ± 350 mg/L, total COD 19800 ± 380 mg/L, specific activity on acetate 0.12 g CH_4-COD/g (VS·d). The PFS used in this study was purchased from Chongqing Reagent Company, which has a total iron value of 20%, and with a residual ferrous content less than 0.1%.

2.2. Methane production during WAS anaerobic digestion in the presence of different PFS levels

Anaerobic digestion of WAS in the presence of different PFS levels was conducted through batch experiments in five replicate serum reactors each with a working volume of 1.0 L. First, each reactor was

fed with 800 mL WAS. Afterward, different volumes of PFS solution (3% w/w) were added into those reactors to achieve the predetermined dosage at the beginning of the experiment, followed by 120 rpm of stirring for 2 min and 60 rpm for 10 min (Zhang et al., 2018; Zouboulis et al., 2008). The predetermined dosages of PFS addition were 0 (control), 5, 10, 20, and 40 g/kg TS, respectively. Next, 400 mL of seed sludge was equally divided and added before the pH of these five reactors was adjusted to 7.0 ± 0.1 with 4 M hydrochloric acid or 4 M sodium hydroxide. Then, each reactor was diluted with Milli-Q water to 1.0 L. To exclude the methane production from seed sludge, one blank reactor contained 80 mL of seed sludge and 920 mL of Milli-Q water was also operated. Oxygen in all the reactors was removed by purging with nitrogen gas for 5 min. After that, all reactors were capped with rubber stoppers, sealed, and incubated at 35 ± 1 ℃ in an air-bath shaker (120 rpm). The pH of all reactors was controlled at 7.0 ± 0.1 in the whole digestion period. The biogas yield, methane and H_2S content in biogas were determined every 2 days. The calculation of the cumulative volume of methane was detailed in our previous publications (Wang et al., 2018). It should be noted that all tests were conducted in triplicate in this study, and the methane production reported were net values with the values measured in blank excluded.

2.3. Effect of PFS on solubilization, hydrolysis, acidogenesis, and methanogenesis of anaerobic digestion

In the digesters operated above, the concentration of soluble protein (carbohydrate) in digestion supernate, VSS reduction and floc size of sludge were analyzed after digestion of 3 days, and by comparing these results, the impact of PFS on solubilization process could be indicated. To assess the effect of PFS on the processes of hydrolysis, acidogenesis, and methanogenesis, the following batch tests using synthetic wastewaters were carried out (Angelidaki et al., 2009; Wang et al., 2018). Twelve reactors with working volume of 1.0 L each were first divided into four groups (Test-I, Test-II, Test-III, and Test-IV) with three in

each. Test-I, Test-II, Test-III, and Test-IV were respectively used to evaluate the impact of PFS on hydrolysis, acidogenesis, acetotrophic methanogenesis, and hydrogenotrophic methanogenesis. All these tests were lasted for 3 d.

Test-I: The three reactors received 920 mL synthetic wastewater and 80 mL of the same seed sludge collected from a laboratory anaerobic sludge digester. The synthetic wastewater contains 6.0 g BSA and 1.2 g dextran. Two reactors were respectively fed with 0.15 and 0.60 g PFS (the amount of PFS is equal to that in the 5 and 20 g/kg TS PFS digester, respectively) while the other reactor received no PFS and was set as the control. All other conditions were the same as those described above. By measuring the degradation rates of protein and dextran, the effect of PFS on hydrolysis process could be indicated.

Test-II: This test was operated the same as that described in Test-I except that the substrates (i.e., BSA and dextran) in synthetic wastewater were replaced by 4.0 g L-alanine (model amino acid compound) and 0.8 g glucose (model monosaccharide compound), respectively.

Test-III: The operation of this test was performed with the same approach as that described in Test-I except that 3.0 g sodium acetate was employed to replace BSA and dextran in synthetic wastewater.

Test-IV: The operation of this test was performed with the same approach as that described in Test-I except that the mixture of standard gas (40% hydrogen, 10% carbon dioxide and 50% nitrogen) was employed to replace BSA and dextran in gas and liquid phase by flushing the configured standard gas for 5 min.

2.4. Identifying the effect of components of PFS on methane production

As mentioned above, the chemical components of PFS can be divided into three types: hydroxyl polymers, iron, sulfate in hydrous media and sediments. The addition of PFS would introduce these three components into the digestion system, which might have different effects on anaerobic digestion. To reveal this question, the following batch test was carried out using real sludge as the digestion substrates. In this

test, 5 replicate serum bottles with identical working volume (1.0 L) were performed, and each received 800 mL WAS. Among them, one was served as the control without addition of any chemical, while the other 4 bottles received 20 g/kg TS PFS, 11.6 g/kg TS ferric chloride, 20.6 g/kg TS potassium sulfate, or 14.3 g/kg TS ferric sulfate, respectively. It should be noted that ferric chloride and potassium sulfate were set as substitute for iron and sulfate to evaluate their effect on methane production, respectively, with their initial concentrations selected based on the stoichiometric content of iron and sulfate in 20 g/kg TS PFS. Because the hydroxyl polymers cannot be modeled, the ferric sulfate was used as a substitute for co-exist of iron and sulfate to qualitatively reflect its effect on methane production.

After the addition of the chemicals, all the bottles were diluted with Milli-Q water to 920 mL and inoculated with 80 mL seed sludge. After flushing with nitrogen gas for 5 min to remove oxygen, all bottles were sealed closely and placed in an air-bath shaker (120 rpm) under 35 ± 1 °C. All other operating steps were the same as those mentioned in Section 2.2.

2.5. Long-Term operation of semi-continuous digesters for microbial activities and community measurement

To reveal the effect of PFS on anaerobic digestion of WAS from the point of microbial activities and community, two semi-continuous digesters were operated in this work. The two reactors were fed with either 0 or 20 g/kg TS PFS-added sludge, with the 20 g/kg TS chosen as the representative dosage of PFS in the actual dewatered-sludge. At the start-up phase (from 0 to the day of maximum methane production occurred in batch tests), the two semi-continuous digesters were operated the same as 0 or 20 g/kg TS PFS-added reactors in Section 2.2, respectively. And then, the sludge retention time in the two digesters was controlled at 20 and 26 d, by withdrawing 50.1 and 38.5 mL of digestion mixtures and replacing with respective same volume of new sludge each day, respectively, based on the results of the batch experiments in

Section 2.2. After 2 months' operation, the daily methane production did not change significantly with time, and then the measurements of key enzyme activities and microbial community were conducted.

2.6. Model-based analysis

Methane production was simulated by the modified Gompertz equation (Eq 1), and kinetic parameters (*Mm*, maximal methane yield potential, mL/g VS or mL/L; *Rm*, maximal methane production rate, mL/(g VS·d) or mL/d or mL/ (L·d); λ, lag-phase time of methane production, d; and t, digestion time, d; *e* is exp(l).) were calculated using Origin 7.0 software (Lay et al., 1997).

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The relationships of PFS concentration with maximal methane yield potential (*Mm*, mL/g VSS or mL/L), maximal methane production rate (*Rm*, mL/ (g VS·d) or mL/ (L·d)) and lag phase time of methane production (λ, d) can be simulated by exponential equations using Origin 7.0 software.

The degradation efficiency of model compounds (e.g., BSA, dextran and butyrate) can be calculated by Eq 2, where C_0 is the initial concentration of model compounds, and C_t is the concentration of model compounds measured at a certain fermentation time (d).

Degradation efficiency (%) = $100 \times (C_0 - C_t)/C_0$ (2)

The specific degradation rates of model compounds are obtained by the zero-order kinetic model Eq 3, where X is degradation kinetics rate (mg/(L·d)) of model compounds (Batstone et al., 2004).

$C_0 - C_t = X \times t$ (3)

2.7. Analytical methods

The measurements of TS, VS, TSS, VSS, COD, soluble carbohydrate, soluble protein, and short-chain fatty acids were in accordance with the Standard Methods and previous literatures (Rice et al., 2012; Xu et al., 2019). The COD conversion coefficients of protein and carbohydrate are 1.5 and 1.06, respectively (Li et al.,

2020a, 2020b). The volumes of biogas were determined by releasing the pressure in the serum bottle using a 300 mL glass syringe to equilibrate with the room pressure according to the literature (Wang et al., 2019a; 2020). The composition of methane and H_2S in biogas was analyzed by gas chromatograph equipped with a thermal conductivity detector according to the method documented in the literatures (Liu et al., 2015; Wang et al., 2019b). Concentrations of aqueous Fe(III) and Fe(II) were determined using by Inductively Coupled Plasma Optic Emission Spectrometer (ICAP 6300, Thermo Fisher Scientific, USA), based on the methods reported previously (Li et al., 2020a). Quantifications of aqueous sulfate and sulfide were performed using Anion Chromatography System with UV and conductivity detector (ICS-900, Dionex, USA) (Dai et al., 2017; Gutierrez et al., 2009). The floc size distribution analysis was performed using a Malvern Mastersizer 2000 instrument with a detection range of 0.01 ~ 3500 μm.

In addition, the activities of protease, acetate kinase, amidase and coenzyme F420 in semi-continuous digesters were measured based on the methods reported previously without major modifications (Du et al., 2020; Fu et al., 2020; Liu et al., 2019). The microbial community in semi-continuous digesters was determined using high-throughput 16S rRNA gene-based IlluminaMiSeq sequencing, with the 515FmodF (GTGYCAGCMGCCGCGGTAA) and 806RmodR (GGACTACNVGGGTWTCTAAT) chosen as PCR primers, and the operational taxonomic units (OTUs) clustered with 97% similarity cutoff (Xu et al., 2020).

2.8. Statistical analysis

All experiments were performed in triplicate, and the results were reported as mean ± standard deviation values. An analysis of variance with least significant difference test was used to assess the significance of results, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Effect of PFS on methane production from anaerobic digestion of WAS

Fig. 1A shows the cumulative methane yield from WAS during anaerobic digestion in the presence of different levels of PFS. After 32 days of digestion, no significant increment of methane production can be found in each digester ($p > 0.05$, Table S1), indicating that the complete anaerobic digestion had been achieved. It can be observed that the optimum digestion time for the digester without PFS addition was 20 d, and at this time the maximal methane yield was obtained (i.e., 144.7 ± 5.0 mL/g VS). Although similar tendencies of methane accumulation were also observed in other digesters with PFS addition, the methane yield was significantly inhibited by PFS. The cumulative methane yield decreased gradually from 91.7 ± 0.3% to 68.0 ± 0.2% of the control with respect to the increasing PFS levels from 5 to 20 g/kg TS and then further decreased to 56.7 ± 0.2% of the control with increasing PFS level to 40 g/kg TS. Further investigation determined that the maximal methane yield showed a well negative correlation with the PFS levels in the sludge ($Y = 152.14 – 3.15\times X + 0.04\times X^2$, $R^2 = 0.9963$, Fig. S2).

To further understand the impact of PFS on anaerobic sludge digestion, the anaerobic digestion kinetics were estimated using the modified Gompertz model (Eq 2). The simulated methane production curves are shown in Fig. 1A, which indicates the fit of methane production to the used model was satisfactory ($R^2 > 0.96$ in all studies cases). Three kinetic parameters, i.e., maximal methane yield potential (*Mm*), maximal methane production rate (*Rm*), and lag phase time (λ), were obtained and showed in Fig. 1B and Table S2. In general, *Mm* and *Rm* decreased exponentially with the increased PFS levels, while the λ presented a reverse tendency. It has been reported that *Rm* is directly relevant to the methanogenic activity, while λ corresponds with the start-up of digester, and both of them depend on the acclimation period of microorganisms to a proper substrate and environmental condition in a batch culture (Batstone et al., 2004; Lay et al., 1997). Thus, it can be concluded that the presence of PFS resulted in the variations of sludge characters and/or digestion system conditions (e.g., solubilization rate, soluble organic components, floc size), which would be further uncovered

in the following text. The above results suggested that the presence of PFS not only decreased biochemical methane potential but also inhibited the rate of methane production and prolonged the start-up period.

3.2. Details of how PFS affects the process of anaerobic digestion

Anaerobic sludge digestion generally contains solubilization of sludge particulate matters, hydrolysis of macromolecular organics (e.g., protein, carbohydrate), acidogenesis of micromolecular organics (e.g, amino acid, glucose), and methanogenesis of acetate and CO_2/H_2 (Fig. S3; Liu et al., 2020a, 2020b; Luo et al., 2020a). The above results indicated that the terminal product of anaerobic digestion, methane, was negatively affected by PFS, but it is still unknown the potential effect of PFS on the four successive bioprocesses. The following analyses were therefore conducted to uncover these gaps.

In this study, the effect of PFS on solubilization process was indicated by comparing the concentration of soluble protein (carbohydrate) in digestion supernate, VSS reduction and floc size of sludge after digestion of 3 days when the solubilized organics from WAS had not been massively bio-consumed for methane production (Fig. S3; Luo et al., 2020b; Wang et al., 2018). As shown in Fig. 2A, both soluble protein and carbohydrate decreased significantly in PFS-added reactors except for the lowest PFS dosage ($p < 0.05$ in all studied cases). And with the increment of PFS addition, the soluble protein and carbohydrate concentration decreased gradually. In the 20 g/kg TS PFS-added reactor, the soluble protein and carbohydrate were respectively 534.2 ± 18.0 mg COD/L and 121.7 ± 5.5 mg COD/L, which were only approximately 66.6% and 69.5% of that in control, suggesting that the addition of PFS inhibited the solubilization process of sludge. This can be further confirmed by VSS reduction (Fig. 2B), an index of specific meaning for sludge solubilization (Wu et al., 2019c; Xu et al., 2019). After 3 days' digestion, the VSS reduction in the control was 13.2 ± 0.5% whereas the corresponding value was only 7.3 ± 0.4% in 40 g/kg TS PFS-added reactor. The higher the PFS dosage, the lower the sludge solubilization, and the less the levels of organic substrates

provided for subsequent methane-producing process (Fig. 2D, R^2 = 0.9822).

The solubilization of sludge is defectively relevant to its floc size (Xu et al., 2020). Fig. 2C illustrates the effect of PFS on the floc size distribution of sludge after digestion of 3 days. The distribution of floc size moved to the increasing direction along with the PFS dosage. The medium diameter of raw sludge was 34.7 ± 2.0 μm. With the addition of 20 g PFS/kg TS for instance, the medium diameter raised to 58.3 ± 2.8 μm, indicating the physical enmeshment increased with the increasing addition of PFS dosage. As a result of physical enmeshment which increased the mass transfer resistance between the organics substrates and microbes (Chu et al., 2005), it can be revealed why the increase addition of PFS inhibited the sludge solubilization process, as well as the methane production (Fig. 2D, R^2 = 0.9764).

The results of batch tests using model organics as substrates were summarized in Table S3 and Fig. S4. It can be observed that the degradations of all tested substrates were affected, and the higher the PFS dosage the lower the degradation efficiency. Fig. 3 shows the specific degradation rates of model compounds obtained by the zero-order kinetic model. It can be seen that the specific degradation rates of BSA, dextran, L-alanine, glucose, acetate, and hydrogen in the control were 1.39, 0.51, 1.02, 0.24, 0.89, and 0.089 L/(L·d), respectively. However, in the presence of 20 g/kg TS PFS, these values decreased to 0.69, 0.27, 0.58, 0.13, 0.49 and 0.040 L/(L·d), and which was 49.6%, 52.9%, 56.9%, 54.2%, 55.1%, and 40.8% of that in control, respectively, suggesting that the process of hydrolysis, acidogenesis, and methanogenesis involved in anaerobic digestion were severely restrained by PFS. As indicated by the correlation analysis in Fig. S5, the degradation rate of these model compounds showed a linear correlation with methane production rate in Fig. 1B with R^2 values higher than 0.97. In addition, the activities of key enzymes (i.e., protease, acetate kinase, and coenzyme F420) in the semi-continuous digesters were detected and the results were out lined in Fig. S6. It can be found that the relative activities of protease, acetate kinase, and coenzyme F420, which respectively

represents the hydrolysis, acidogenesis, and methanogenesis processes in 20 g/kg TS PFS-added digester, was 62.8%, 70.5%, and 57.3% of that in control digester. Thus, it can be speculated that the inhibited enzymes activity was one reason for the restrained bio-processes (Fig. 3) and the decreased methane production rate (Fig. 1B). Our previous publications indicated that the polymers such as PAM, an organic macromolecular flocculant, would cover both organic substrate and anaerobic microbes through its chemical characteristics, resulting in the deterioration of normal enzyme metabolism (Wang et al., 2018). The hydrolyzed PFS might play a similar role in the batch digester. Besides the chemical effect, the main derivatives of PFS such as Fe(III) and sulfate as well as their aggregations, would act as the electron acceptor and cause alterations of anaerobic biological processes, which were explored in the following analyses.

Fig. 4 shows the profile curves of aqueous iron and sulfate (sulfide) in the 20 g/kg TS PFS-added digester. Dissolved Fe(III) and sulfate decreased gradually along with digestion time, indicating the iron and sulfate reduction during anaerobic digestion of WAS (Yu et al., 2018). Correspondingly, the Fe(II) increased substantially from 0.1 ± 0.0 mg/L to 2.6 ± 0.1 mg/L after 4 days of anaerobic digestion, and the sulfide rose piecemeal in the whole digestion time (0.7 ~ 3.5 mg S/L), indicating the reduction of iron and sulfate which were driven by iron and sulfate reduction bacteria (IRB and SRB), respectively (Liu et al., 2015). However, the Fe(II) concentration peaked on day 4 and then decreased immediately to nearly zero on day 16, which showed an inverse tendency with that of sulfide and could be attributed to the precipitation of Fe(II) with sulfide. Based on the preliminary balance analysis of iron and sulfur in the anaerobic digestion system, it can be found that most of them were precipitated and/or adsorbed in sediments (> 80%). Besides the behavior occurred in liquid phase, there might exist some biological or chemical behaviors of PFS and its derivates in sediments that affecting methane-producing process (Van Den Berg et al., 1980; Zeng et al., 2020), which required to be further investigations in the future.

Fig. 4C further illustrates the H_2S production in control and PFS-added reactors during anaerobic digestion. In the initial stage of anaerobic digestion (0 ~ 14 days), the H_2S production in PFS-added reactors were all lower than that of control. The inhibited production of H_2S could be attributed to the mass transfer resistance between the sulfate and sulfate reducing bacteria caused by PFS and the precipitation of sulfide with Fe(II). However, as anaerobic digestion progressed, the cumulative H_2S production in PFS-added reactors exceeded that of control. After 32 days of digestion, the cumulative H_2S production in the control was $93.2 \pm 4.8 \times 10^{-4}$ mL/g VS, and the cumulative production in the 20 and 40 g/kg TS PFS-added reactor was respectively $107.3 \pm 5.5 \times 10^{-4}$ and $118.7 \pm 6.0 \times 10^{-4}$ mL/g VS, which was 115.1% and 127.4% of the control. In Fig. 4B, the concentration of sulfate in liquid phase showed no significant decrement after digestion of 14 days, indicating that the occurrence of sulfate reduction in the solid phase and might be attributed to the reduction of sulfur-containing compounds and the precipitated sulfate substance deserved from PFS as well. It should be noted that H_2S might have an inhibitory effect on some methanogenic species, which might lower the methane production, because the used seed sludge was not sulfate-acclimatised in present study (Cetecioglu et al., 2019), and this might be another reason for the decreased methane production in Fig. 1. The above results indicated that the addition of PFS in sludge increased the H_2S production during anaerobic digestion, which might damage methanogens in anaerobic digestion system and cause detrimental problems such as malodors, health hazards and corrosion, need to be taken seriously (Zeng et al., 2020).

3.3. Identifying the effect of components of PFS on methane production

Addition of PFS would introduce three components (i.e., hydroxyl polymers, iron, sulfate) into the digestion system. The different components of PFS might have different effect on sludge anaerobic digestion, thus their respective impacts on methane yield were identified in this work. Fig. S7 illustrates the methane production from the components-added reactors along with the digestion time, with the

corresponding kinetic parameters of each case shown in Fig. 5A-6C. It was clearly observed that all of these components from PFS significantly affected the methane production during anaerobic digestion of WAS ($p < 0.05$ in all studied cases).

The potassium sulfate, as the substitute of sulfate from PFS, showed a 17.5 ± 0.1% inhibition on methane production. As summarized in Fig. 5D, the sulfate could induce the enrichment of SRB and the competition of organic substrates with methanogens, as well as the production of hydrogen sulfide in anaerobic digestion systems (Fig. 4C), which could diffuse across cell membranes and result in protein denaturation and enzyme inactivation, thereby deteriorate the anaerobic digestion for methane production (Ge et al., 2013; Wei et al., 2018b; Yuan et al., 2016). This study further confirmed these conclusions. Similarly, the addition of related dose of ferric sulfate led to a 11.1 ± 0.1% decrement of methane production. The reason for this result might be that the iron content was inadequate for sulfate precipitation.

In the presence of ferric chloride, as the substitute of iron from PFS, the methane production of WAS increased approximately 17.6 ± 0.1 % compared with that of the control. This result was accorded with the favorable effect of iron on the anaerobic digestion process previously reported, and as shown in Fig. 5D, the improvement could be attributed to two aspects: i) being an essential component of microbial cells, and ii) serving as an accelerator for the hydrolysis-acidification process and biodegradable organic matters generation (Liu et al., 2015; Wei et al., 2018b; Yu et al., 2015a, 2015b). Compared to PFS, the iron-equivalent ferric sulfate showed less inhibition (11.1% versus 32.0%), and this gap might be ascribed to the “hydroxyl polymers” to some extent. Our previous studies demonstrated that the organic polymer, polyacrylamide, would induce the aggregation of sludge flocs and increase the mass transfer resistance between the microbes and organic substrates, thereby deteriorated the anaerobic digestion performances (Wang et al., 2018). It is worth noting that the hydrolysis of ferric chloride also generates “hydroxide polymers”, however, such species

are generally scattered in liquid/solid phase at neutral conditions and easily decomposed by anaerobic microbes and further utilized for metabolism (Qin et al., 2019; Yu et al., 2015), whereas the PFS-derived "hydroxide polymers", could be expressed as $(Fe_iO_k(OH)_{ij-2k})^{i(3-j)}$ and "multinucleate and multichain-hydroxyl polymers", have more nucleus, longer cross-linked chains, punchier aggregation, ~15 times higher coagulation efficiency ((Jiang et al., 1998; Zouboulis et al., 2008), might play a similar "destroyer" role toward anaerobic digestion.

In addition, based on the variance analysis results within these cases (Fig. 5A-5C), it can be further demonstrated that among the main components and/or forms of PFS present in anaerobic digester, "multinucleate and multichain-hydroxyl polymers" and sulfate were unveiled to be the major contributors to the decreased biomethane potential ($p < 0.02$), and the "multinucleate and multichain-hydroxyl polymers" was the main buster to the slowed methane-producing rate ($p < 0.02$) and the extended lag time ($p < 0.01$). It should be noted that the present identification was based on the stoichiometric composition of iron and sulfate in PFS, with the physical differences between model reagents and the targeted derivates being ignored. More works on the detailed biological or chemical behaviors and balance of sulfur and iron in the PFS-added digestion system, including gas, liquid, and solid phase, should be carried later, although the present results could provide an insight into the effect of components of PFS on methane production to some degree.

3.4. Effect of PFS on microbial community in long-term operation of semi-continuous digesters

The microbial community was measured through high-throughput 16S rRNA sequencing to compare the community diversity, structure, and function in semi-continuous digesters. Fig. S8 illustrates the methane yield of the two semi-continuous digesters, which further confirmed that the presence of PFS inhibited the anaerobic methane production. It can be found that after 2 months' operation, the methane yield in the two digesters did not change significantly with time, indicating a stable state for microbial community

measurement. Ecological diversity indices of the bacteria and archaea, the Simpsoneven, Simpson's E and Shannon's H indices, decreased in the PFS-added reactor (Fig. 6A). In the presence of 20 g/kg TS PFS, the richness, evenness and diversity metrics of 0.97 OTUs decreased from 1800 ± 154, 5.767 ± 0.620 and 0.063 ± 0.013 to 1466 ± 130, 0.050 ± 0.009 and 5.214 ± 0.480. The possible reason for this effect might contribute to the biotoxicity of PFS to anaerobic digestion process. The results can be further revealed by a bipartite association network showing the associations of bacteria and archaea in each digester at genus level (Fig. 6B and 7C). It can be found that of these species, 88.7% bacteria and 100% archaea in PFS-added digester were associated with the control, indicating that the basic similarity of the communities in the two digesters. However, at PFS-added digester, the exclusive species regardless of bacteria and archaea were significantly lower than that of the control digester. These results suggest that the presence of PFS can reduce microbial alpha diversity of digester.

Sunburst plot based community analysis was carried out to reveal how the PFS affected the community structure of bacteria and archaea from the phylum level to genus level (Fig. 7). For the bacteria community posted in Fig. 7A, the *Chloroflexi*, *Bacteroidetes* and *Proteobacteria*, were the dominant phyla in two detected digesters regardless of the presence or absence of PFS, and no new dominant species emerged in the PFS-added digesters, which were consistent with the results outlined in Fig. 6. The predominant genera of bacteria were *vadinHA17* sp, *Anaerolineaceae_norank* sp, and *Longilinea* sp, which respectively affiliated to phyla *Bacteroidetes*, *Chloroflexi*, and *Chloroflexi*., in the presence of 20 g/kg TS PFS, varied much from that of the control in relative abundances. The *Anaerolineaceae_norank* sp and *Longilinea* sp, which were reported to have ability to degrade some carbohydrates and be associated to syntrophic communities of bacteria and methanogenic archaea (Xu et al., 2017; Yamada et al., 2007), decreased to 7.7% and 8.8% in 20 g/kg TS PFS-added digester (13.1% and 12.7% in control digester), respectively. Similar trends could be

found in genera *Anaerolineaceae_noclassified* sp, *Leptolinea* sp, and *BD1-7-clade* sp, which have been documented to be hydrocarbon degraders and main participators in hydrolysis-acidification process (Kim et al., 2012; Liu et al., 2019), indicating that the PFS caused the shift of the microbial structure toward the direction against hydrolysis and acidogenesis, which were in agreement with the deteriorated performances in hydrolysis and acidogenesis model experiments (Fig. 3). The *vadinHA17* sp, which was usually found in the environment rich in refractory and/or complex organics such as pectin and hemicellulose, accounted for 20.3% in the 20 g/kg TS PFS-added digester, whereas this value was only 13.2% in the control, was assumed to be the despondence for the formed multinucleate and multichain-hydroxyl polymers derived from PFS (Liu et al., 2019; Nelson et al., 2011). Specially, the genera of *thermodesulfovibrio* sp, belonged to phyla *Nitrospirae*, and is capable of sulfate and or iron reducing, was enriched with the presence of PFS (3.04% versus 1.97%), reflecting that the PFS stimulated the sulfate and/or iron reducing bacteria during anaerobic digestion (Sekiguchi et al., 2008).

Regarding the archaeal community, the predominant genera of methanogens were *Methanosaeta* sp, *Methanobacterium* sp, *Candidatus_Methanofastidiosum* sp, and *Methanolinea* sp, which accounted for 47.2%, 21.9%, 11.9%, and 6.65% of archaea 16S rRNA gene sequences of the control (Fig. 7B). In the presence of PFS (e.g., 20 g/kg TS), the structure of predominant genera of methanogens did not differ much from that of the control, but their relative abundance shifted largely. The genera of *Methanobacterium* sp, *Candidatus_Methanofastidiosum* sp and *Methanolinea* sp, which have been proved to be hydrogenotrophic methanogens (He et al., 2019; Sakai et al., 2012), dropped to 11.5%, 7.83%, and 3.13%, respectively. The genera of *Methanosaeta* sp, the only known strict acetoclastic methanogen (Narayanan et al., 2009), increased significantly with the addition of 20 g PFS/kg TS (from 47.2% to 68.4%), and the *Woesearchaeia_norank* sp, has the ability to use H_2 to reduce methyl compounds to produce CH_4 (Gründger et al., 2019), also increased

to 3.93%. These facts suggested that the pathway of CH_4 production was partially transferred from hydrogenotrophic to acetotrophic methanogenesis, which further confirmed that the presence of PFS showed a severer inhibition on hydrogenotrophic methanogens than acetotrophic methanogens, as shown in Fig. 2C. Previous studies demonstrated that the diffusion of gas in polymer solutions is far less effective than low-molecular organics, thus it can be speculated that the severe mass transfer resistance of H_2 between the syntrophs and methanogens caused by the formed multinucleate and multichain-hydroxyl polymers of PFS were the contributors to the transferred methanogenesis pathway and inhibited hydrogenotrophic methanogens (Caulfied et al., 2002; Kawashima et al., 1990). In addition, as shown in Fig. 7A, PFS stimulated the sulfate and/or iron reducing bacteria during anaerobic digestion (i.e., *thermodesulfovibrio* sp). The genera of *thermodesulfovibrio* sp, was proven to be a generalist participating in the metabolism of anaerobic intermediates and use pyruvate, and hydrogen as electron donors in the presence of sulfate (Liang et al., 2016). And more importantly, they have a higher appetency for hydrogen than methanogens in the case of insufficient/limited hydrogen content, which might be the other reasons for the shift and/or differences deciphered above (Harada et al., 1994).

3.6. Implications for wastewater and sludge treatments

This study investigated the effects of PFS on anaerobic digestion of WAS and underlying mechanisms for the first time, through batch and long-term tests using either synthetic wastewaters or real WAS as the digestion substrates. It was found that the presence of PFS resulted in a terrible performance of anaerobic sludge digestion. From macro to micro levels, it was revealed that the physical enmeshment caused by PFS significantly lessened the levels of organic substrates for methane-producing process, the presence of PFS severely restrained the anaerobic bio-processes of hydrolysis, acidogenesis and methanogenesis by lowering down the activities of the relevant enzymes, and meanwhile, the produced or surplus sulfide from PFS also

had a toxicity on methanogens (Fig. 8), which could be confirmed by microbial community results. Especially, PFS's inhibitions to hydrogenotrophic methanogenesis was much severer than that to acetotrophic methanogenesis, which could be further supported by the elevated abundances of *Methanosaeta* sp and the dropped abundances of *Methanobacterium* sp in PFS-added digester, and probably due to the severe mass transfer resistance of hydrogen between the syntrophs and methanogens caused by PFS, as well as the higher hydrogen appetency of PFS-induced sulfate reducing bacteria.

Considering the negative effects of PFS on anaerobic sludge digestion, more attentions should be paid when using in wastewater management and sludge treatments systems, to avoid its entrance to the WAS anaerobic system. Although the use of flocculants such as PFS is inevitable, there are two optimal solutions to lower down the possibility of PFS entering sludge anaerobic system. One is to compound with other no or less toxicity flocculants such as ferric chloride, chitosan, starch, and other microbial flocculants, by which the usage amount of PFS could be significantly reduced in flocculants process (Wei et al., 2018a; Yu et al., 2015). The other one is to lessen the percentage of sulfate through modifying the PFS using some natural compounds such as sodium alginate and chitosan (Wu et al., 2019a, 2019b). The sewage and sludge treatment should be coupled as a whole, one should not consider only the performances enhancement of sewage pretreatment and/or sludge dewatering by using plenty of flocculants and ignore the flocculant's potential effect to other biochemical processes, such as biological nitrogen and phosphorus removal, anaerobic digestion. In addition, the physical enmeshment caused by PFS and high TS of sludge was found to be one of the reasons for the decreased methane production, thus, it can be speculated that diluting the PFS-contained sludge might be an effective way for mitigating PFS's negative effects. However, it is noticeable that this work mainly aimed to provide insights into the PFS-involved anaerobic digestion system, more effects should be devoted to mitigating PFS's negative effects in the future.

4. Conclusion

This study revealed the effects of PFS on anaerobic digestion of WAS and underlying mechanisms for the first time. The main conclusions are: (1) The presence of PFS not only decreased biochemical methane potential but also inhibited the methane production rate and prolonged the start-up period during anaerobic sludge digestion. (2) PFS significantly lessened the levels of organic substrates for methane-producing process, and meanwhile severely restrained the anaerobic bio-processes of hydrolysis, acidogenesis and methanogenesis. (3) PFS decreased the microbial diversity and caused the shift of microbial structure toward the direction against hydrolysis, acidogenesis and methanogenesis. (4) "multinucleate and multichain-hydroxyl polymers" and sulfate from PFS were unveiled to be the major contributors to the decreased biomethane potential, and the "multinucleate and multichain-hydroxyl polymers" were the chief buster to the slowed methane-producing rate and the extended lag time. The findings obtained herein would make a sound contribution to the mitigation of PFS's negative effects in wastewater and/or WAS treatment system in the future.

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Appendix A. Supplementary data

This file contains analytical methods, Table S1 – S3 and Fig. S1 – S8.

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Fig. 1. Cumulative methane production during WAS anaerobic digestion in the presence of different levels of PFS (A), and correlation between PFS addition and the maximal methane yield potential, maximal methane production rate and lag phase time from model fit (B). Symbols represent experimental measurements and curves represent model fit. Error bars represent standard deviations of triplicate tests.

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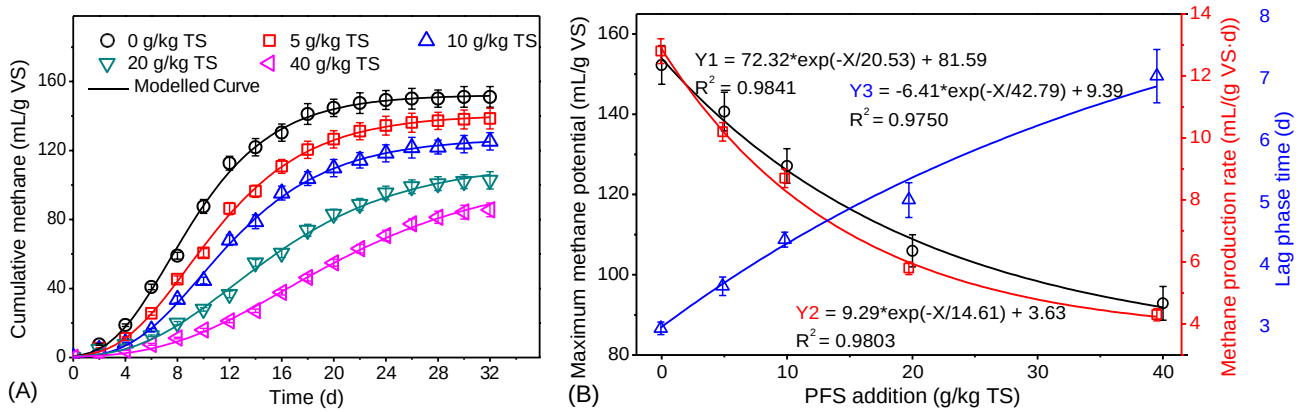
Figure



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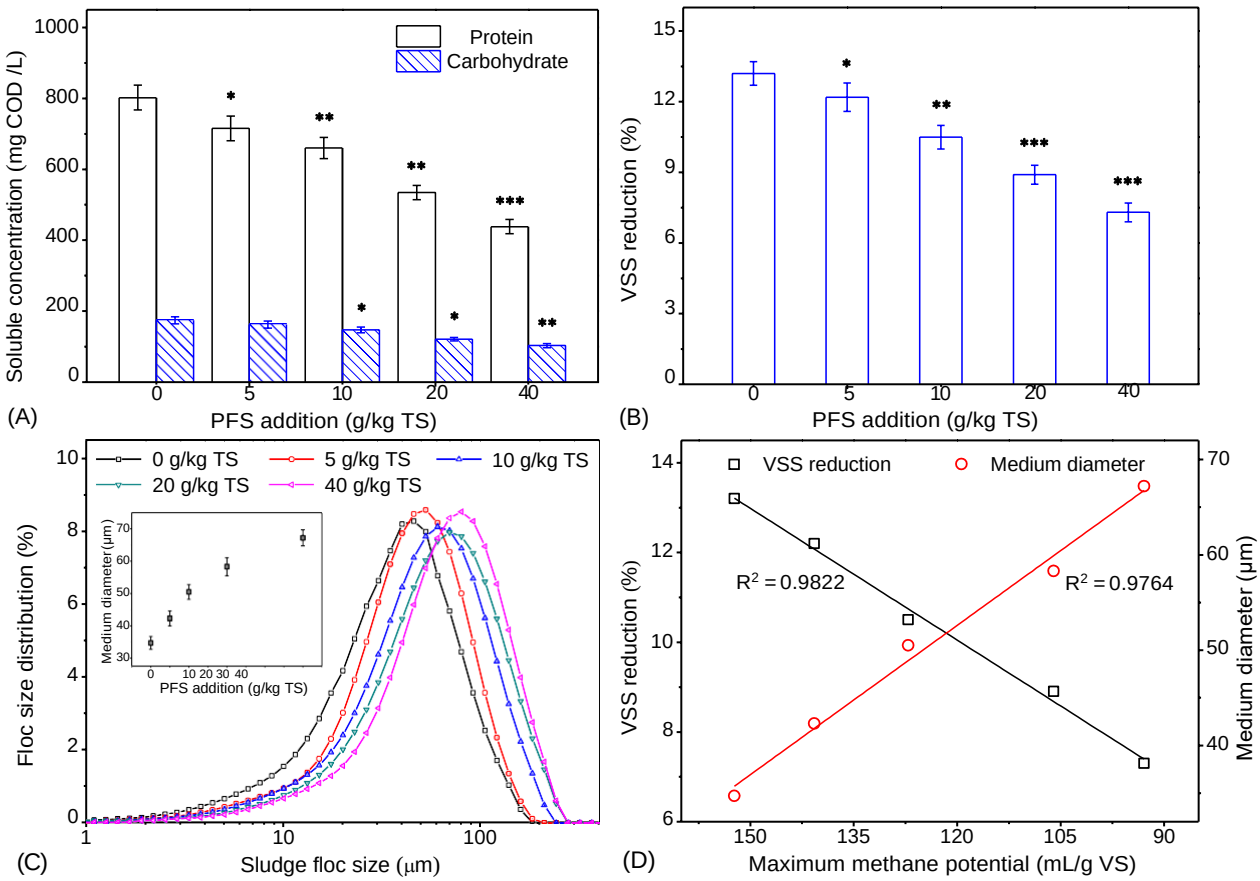

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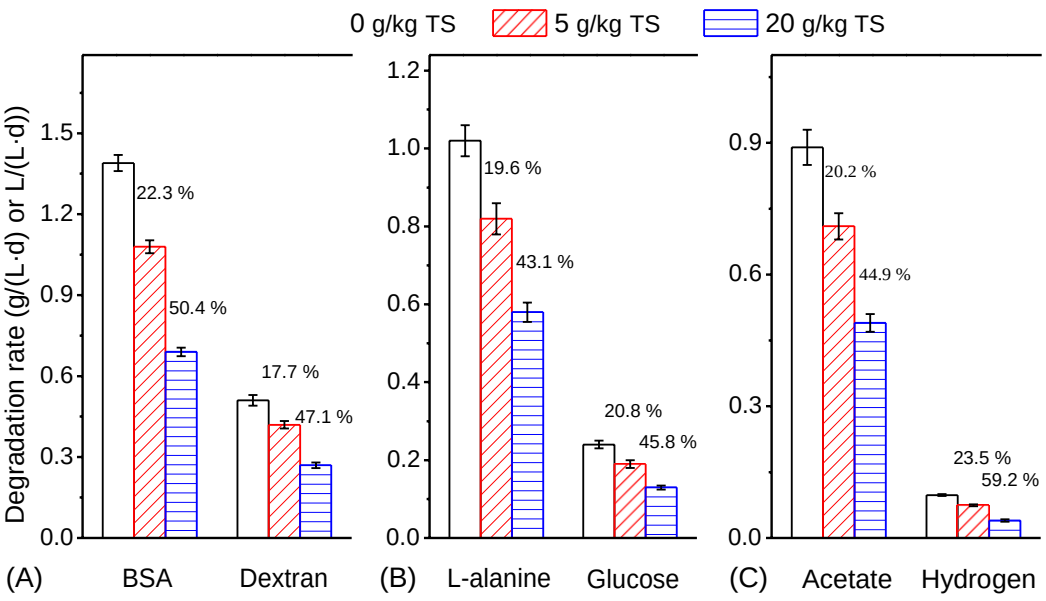

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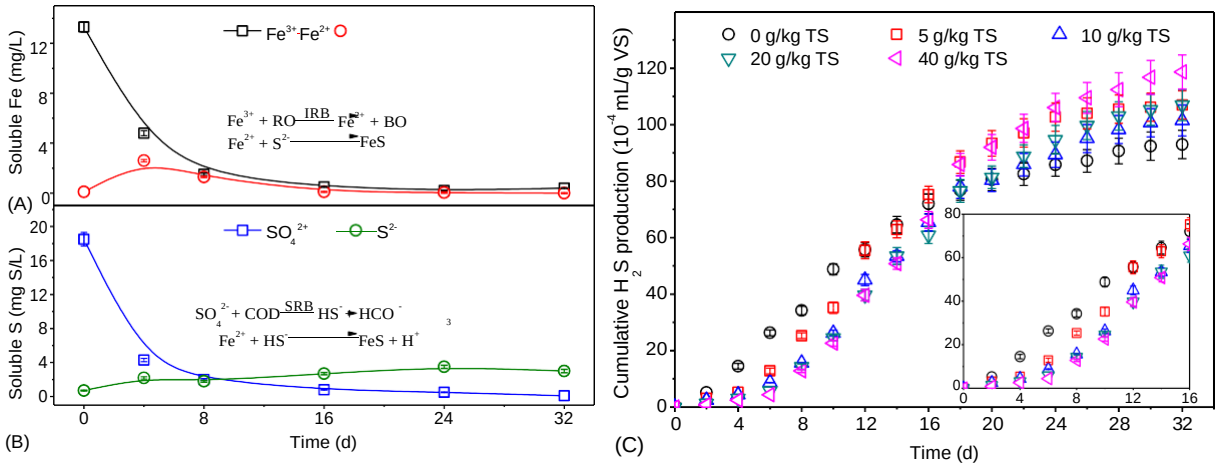

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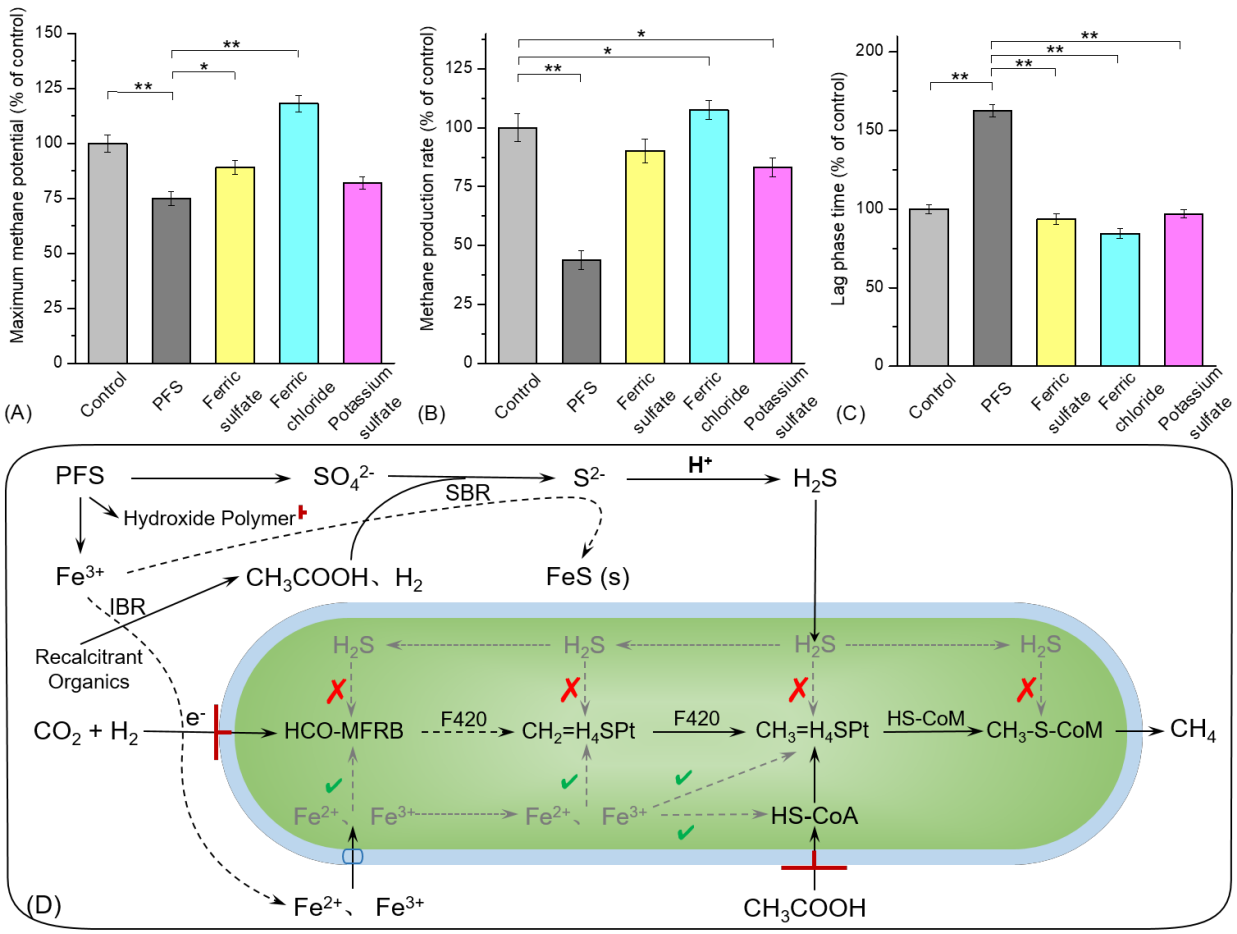

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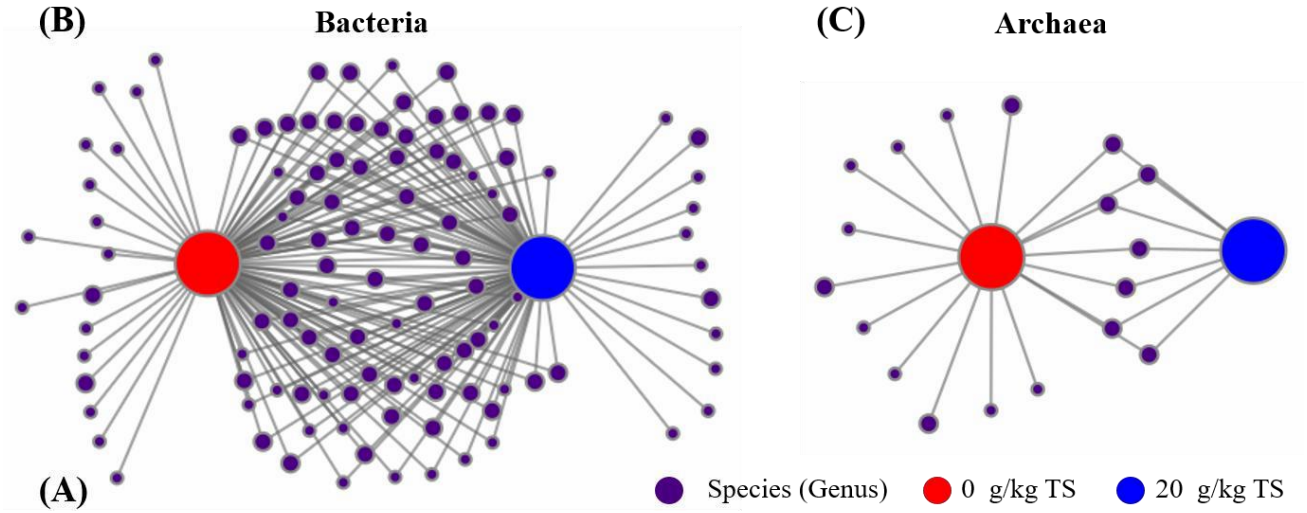

Digester (g/kg TS)	Richness (observed sp.)	Evenness (Simpson's E)	Diversity (Shannon's H)
0	1800 ± 154	0.063 ± 0.013	5.767 ± 0.620
20	1466 ± 130	0.050 ± 0.009	5.214 ± 0.480

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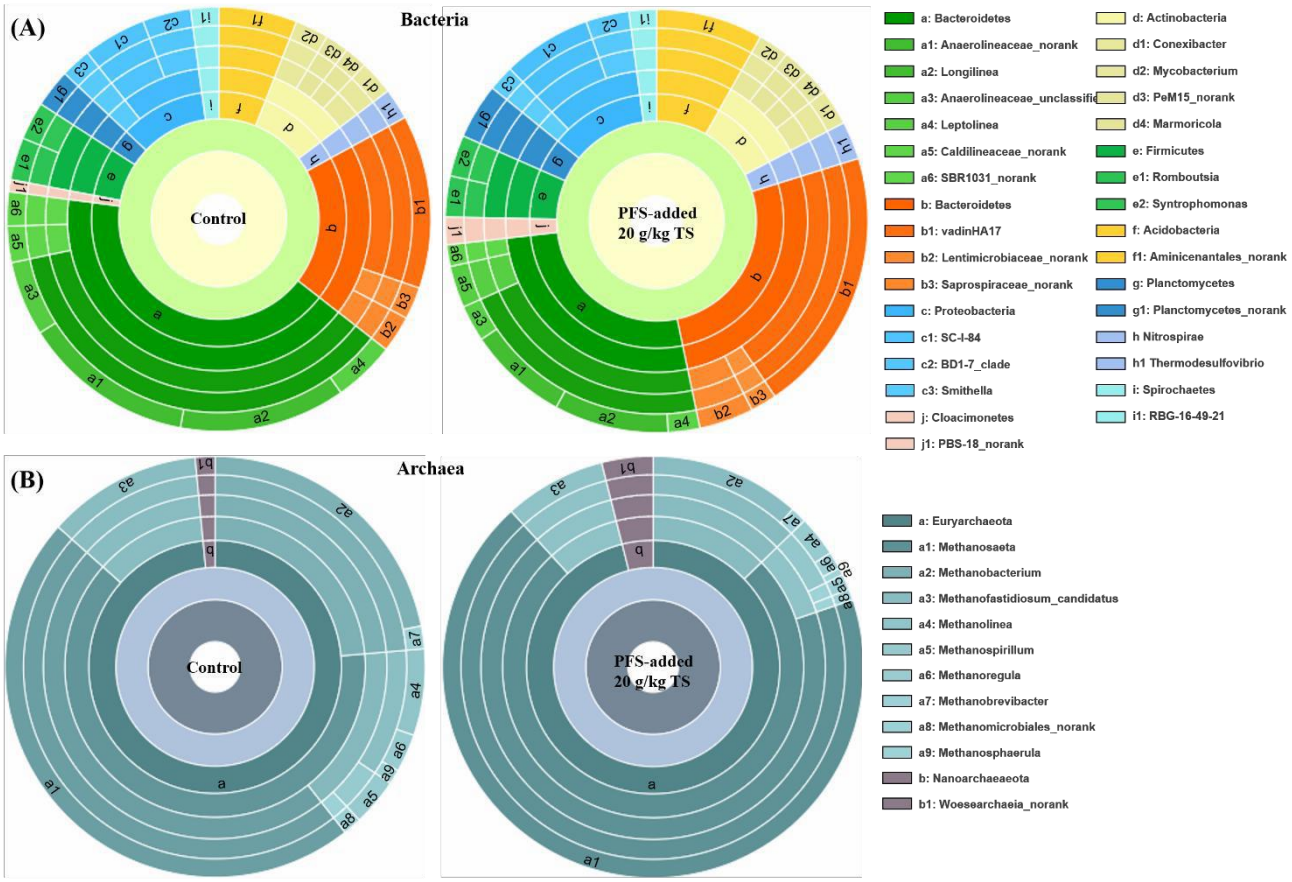

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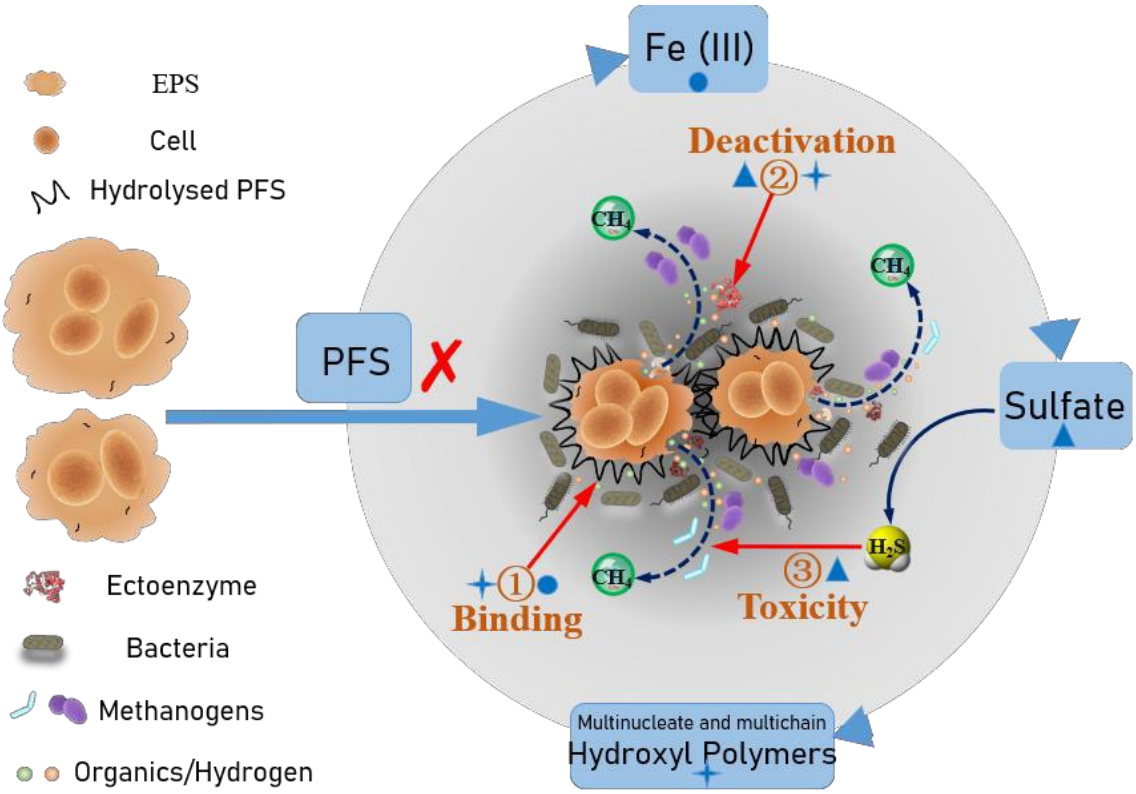

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