Digestion Liquid Based Alkaline Pretreatment of Waste Activated Sludge Promotes Methane Production from Anaerobic Digestion

Dandan He[a,b], Jun Xiao [a,b], Dongbo Wang[a,b*], Xuran Liu[a,b], Qizi Fu [a,b], Yifu Li [a,b], Mingting Du [a,b], Qi Yang[a,b], Yiwen Liu[c], Qilin Wang[c], Bing-Jie Ni[c], Kang Song [d*], Zhe Cai[e], Jun Ye[e], Haitao Yu[e]

[a]College of Environmental Science and Engineering, Hunan University, Changsha 410082, P.R. China

[b]Key Laboratory of Environmental Biology and Pollution Control, Ministry of Education, Changsha 410082, P.R. China

[c]Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney, Sydney, NSW 2007, Australia

[d]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

[e]Hunan Qing Zhi Yuan Environmental Protection Technology Co., Ltd, Changsha 410004, P.R. China

First author

Email: H_Dandan@163.com

*Corresponding author

Email: w.dongbo@yahoo.com (Dongbo Wang); sk@ihb.ac.cn (Kang Song)

ABSTRACT

This work proved an efficient method to significantly increase methane production from anaerobic digestion of WAS. This method is to reflux proper of digestion liquid into waste activated sludge pretreatment unit (pH 9.5 for 24 h). The yield of maximum methane improved between 174.2 ±7.3 and 282.5±14.1 mL/g VSS with the reflux ratio of digestion liquid increasing from 0% to 20%. It was observed that the biodegradable organics in the digestion liquid did not affect the biological processes related to anaerobic digestion but increased methane production through reutilization. The ammonium in the digestion liquid was the main contributor to the increase in methane production via promoting sludge solubilization, but refractory organics were the major inhibitors to anaerobic digestion. It should be emphasized that the metal ions present in the digestion liquid were beneficial rather than harmful to the biological processes in the anaerobic digestion, which may be connected with the fact that certain metal ions were involved in the expression and activation of key enzymes. In addition, it was found that anaerobes in digestion liquid were another potential contributor to the enhanced anaerobic digestion.

Keywords: Digestion liquid; Anaerobic digestion; Alkaline pretreatment; Free ammonia

Introduction

Activated sludge process is currently the most efficient biological wastewater treatment technology being widely used in wastewater treatment plants (WWTPs). But it is inevitable to produce a large amount of activated sludge every day (Li et al., 2020). According to the reports, the annual output of WAS with a moisture content of 80% in China will exceed 60 million tons (Li et al., 2019a; Yang et al., 2015). Untreated WAS is likely to pose a threat to ecosystems, thereby affecting environmental safety and public health (Li et al., 2019; Liu et al., 2020b; Xu et al., 2019). Anaerobic digestion can lead to the reduction and stability of WAS, the killing of pathogenic microorganisms, and the energy recovery in the form of biogas, so it is widely

40 used in WWTPs around the world (Liu et al., 2020; Niu et al., 2019).

41 In ordinary anaerobic digestion, the reduction of WAS is usually less than 30%, which is only equivalent
42 to 5-7% of the available energy in the original sludge (Lazarova et al., 2012). The resource recovery rate of
43 anaerobic digestion is mainly limited by the biochemical methane potential and hydrolysis rate of the substrate,
44 which generally perform poorly in WAS (Li et al., 2017; Liu et al., 2019a). In order to obtain as large
45 methane production as possible, before anaerobic digestion, proper pretreatments are adopted to improve the
46 biochemical methane potential and hydrolysis rate of WAS. So far, numerous WAS pretreatment methods
47 have been proposed, such as thermal, alkaline and chemicals (Wang et al., 2019; Li et al., 2013; Tanaka et al.,
48 1997; Valo et al., 2010). For example, Tanaka et al. (1997) showed that pretreatment of sludge at 180℃ for
49 60 minutes increased methane production by about 90% and VSS degradation rate by 30%, as compared with
50 the blank. Wang et al. (2019) indicated that calcium peroxide pretreatment can reduce the inhibition of
51 refractory organics such as humus and lignocellulose, and increase methane production by 1.5 times. After
52 being decomposed with 0.1 mol/L NaOH, the content of soluble organic matter in WAS was significantly
53 increased, and the biogas production was increased by 33% in comparison with the control (Li et al., 2013).
54 Although the above strategies are effective, they require a large amount of energy or reagents, which increases
55 their cost in actual management.

56 Free ammonia (FA), the non-ionized form of ammonium, can enter cells through passive diffusion,
57 causing cell depression or even death (Fux et al., 2006; Vadivelu et al., 2007). Wei et al. (2017) showed that
58 when the FA concentration was 420-680 mg NH_3-N/L, the biochemical methane potential and hydrolysis rate
59 increase from 160 L CH_4/kg VS and 0.22 d^{-1} to 195 L CH_4/kg VS and 0.53 d^{-1}. Mechanism studies revealed
60 that the primary cause for the increase in methane production was that FA pretreatment enhanced the
61 degradation of WAS and improved the biodegradability of WAS (Wang et al., 2018). In addition, compared

62 with the free nitrous acid pretreatment technology that requires an additional nitrification reactor to be
63 constructed in the side stream to biologically convert ammonium into nitrite, the FA-based technology does
64 not require any additional reactors because FA can be obtained in situ from the digestion liquid with abundant
65 ammonium (Wei et al., 2017). Therefore, FA technology has economic advantages that cannot be ignored,
66 which gained great attraction and expectations in practical applications.

67 There have been some achievements that have boosted the FA technology to a certain extent (Calli et al.,
68 2005; Belmonte et al., 2011; Liu et al., 2019; Wang et al., 2018; Wei et al., 2017), but the FA-based
69 technology in all the previous researches originated from the chemical solution prepared by NH_4Cl, rather
70 than the actual digestion liquid. As we all know, in addition to ammonium, the real digestion liquid also
71 contains various substances. Some of them are biodegradable, such as acetic, protein and carbohydrates,
72 while others are non-biodegradable and may inhibit microorganisms such as humic acid, lignin and metal ion
73 (Mudhoo and Kumar 2013; Sawatdeenarunat et al., 2015; Xiao et al., 2018). As a result, the implementation
74 of FA-based technology in the real project is full of indeterminacy. Specifically, the residuary proteins,
75 carbohydrates, fatty acids and other substances in the digestion liquid may be reused by microorganisms in the
76 digestion system to increase methane production. However, the presence of refractory organics or metal ions
77 was shown to reduce the activity of anaerobic bacteria (Appels et al., 2010; Li et al., 2014), and their
78 coexistence may lead to the increased inhibition, which make against to anaerobic digestion. Up to now, it is
79 doubtful whether reflux of actual digestion liquid can increase methane production.

80 Hence, this study aims to determine whether an appropriate amount of digestion liquid reflux can
81 promote the energy recovery of WAS anaerobic digestion. First of all, the influence of the digestion liquid
82 with different reflux ratios (V/V, 0, 5, 10, 15, 20 and 25%) was investigated. Afterwards, the facts of how
83 digestion liquid affects methane production were clarified by studying its performance in the solubilization of

84 sludge, the biodegradability of organics released and other anaerobic digestion processes. Finally, in order to
85 identify the contribution of the main substances to the enhanced methane production, a series of anaerobic
86 digestion batch tests were also carried out. As far as we know, this is the first work to demonstrate the
87 feasibility of actual digestion liquor promoting methane production from WAS anaerobic digestion, and reveal
88 the mechanism of how digestion liquid affects methane production.

89 Materials and Methods

90 **Raw WAS, Inocula, and Digestion Liquid.** The sludge used for the digestion substrate in this study
91 was the WAS harvested from the second settling tank of a WWTP in Changsha, China. After the sludge was
92 collected, it was placed in a refrigerator at 4 ℃ and allowed to stand for 24 hours. After being discard part of
93 the supernatant, the concentrated sludge was sieved with a 20-mesh sieve for use. The inocula were
94 collected from a long-term anaerobic digester that operated in our lab. Sludge digestion for producing
95 digestion liquid was conducted at pH 9.5 according to the procedure described previously (Wang et al., 2018).
96 After digestion, the liquid phase was separated, the phosphorus was removed as struvite, with the detailed
97 operation being described in Supporting Information (Text S1). The main characteristics of the WAS,
98 inocula and supernatant digestion liquid are listed in Table 1.

99 **Digestion Liquid Pretreatment on WAS.** The 2.7 L of WAS prepared above was divided equally into
100 six bottles. Then, 0 to 150 mL digestion liquid was added to these bottles to achieve the preselected ratio of
101 0, 5, 10, 15, 20 or 25% of digestion liquid to substrate (i.e., raw WAS). Milli-Q water was then injected so
102 that each bottle of digestion substrate was 600 mL. In order to form FA and increase methane production, all
103 the bottles were pretreated with alkaline (pH 9.5) for 24 h, as in our previous report (Wang et al., 2018).

104 **Batch Biochemical Methane Potential Evaluation.** At the end of pretreatment, all the bottles were
105 purged with 200 mL of above inocula and adjusted the pH to neutral (7.0±0.1). Each bottle was flushed with

106 nitrogen gas for 2 min to remove oxygen, then rubber stoppers were used to seal the bottles, which were
107 finally placed in a constant temperature incubator at 35 ± 1 ℃ with 120 rpm. In addition, a blank reactor was
108 set up to eliminate the influence of the inoculum on methane production, which only contained the same
109 volume of inocula and Milli-Q water. The methane production was determined by multiplying the bio-gas
110 volume by the methane concentration in the biogas, and it was a cumulative value according to the previous
111 method (Wang et al., 2019; Logan et al., 2002). The first-order kinetic model was applied to fit the methane
112 production gained above to evaluate the methane production potential and kinetics of WAS, with the details
113 being showed in Supporting Information (Text S2).

114 **Methane Production from the Reflux Digestion Liquid.** In order to eliminate the interference of
115 organics in the reflux digestion liquid on methane production, the following experiments were carried out.
116 Six reactors similar to the above were used in this experiment. The procedure of this test was the same as the
117 section of "Batch Biochemical Methane Potential Evaluation", except that Milli-Q water instead of the
118 alkali-treated WAS was used.

119 **Effect of Major Components in the Digestion Liquid on Methane Production.** As mentioned
120 above, the components of digestion liquid are very complex, which can be divided into biodegradable
121 organics, refractory organics, ammonium, metal ions and microorganisms according to their chemical and
122 biological properties. So as to investigate the effects of these substances on methane production, the
123 following trials were carried out to assess their impacts on the performance of anaerobic digestion. These
124 trials were labeled as Test-A, Test-B, Test-C, Test-D, and Test-E, with the operational procedures being
125 detailed in Supporting Information (Text S3).

126 **Effect of Digestion Liquid and Its Main Components on Anaerobic Digestion Processes.** There
127 are some bio-processes, such as hydrolysis, acidogenesis, acetogenesis, and methonogenesis, in WAS

anaerobic digestion that are closely related to methane production (Madsen et al., 2011). The digestion liquid will inevitably enter the digestion system and contact with the inocula. Therefore, the purpose of the following batch tests was to evaluate the influence of digestion liquid and its main components on each bio-process of anaerobic digestion. In this batch tests, 30 repeated batch reactors were operated. These reactors were divided into five tests (i.e., Test Ⅰ, Test Ⅱ, Test Ⅲ, Test Ⅳ, and Test Ⅴ). The operation procedure was introduced in Supporting Information (Text S4).

Analytical Methods. Hydrogen and methane were analyzed using a gas chromatograph (GC112A, China) with a thermal conductivity detector and a 4 mm × 2 m GDX-102 stainless column (Liu et al., 2021). Soluble proteins and carbohydrates were determined by Lowry-Folin method and phenol-sulfuric acid method. SCFA was quantified with an Agilent 6890N with DB-MAXETR column according to the method detailed in the literature (Wang et al., 2019). The determinations of COD, TSS, NH_4^+-N and VSS were performed on the basis of the standard method (APHA 1998). The determination methods for humus and lignocellulosic were the same as described in the previous literature (Wang et al., 2019; Van et al., 1991). The changes of liquid in pretreated WAS were characterized by using an Excitation Emission Matrix luminescence spectroscopy (F-4600 FL spectrophotometer Hitachi Japan), and the specific operational procedure can be found in Text S5 (Supporting Information). The inductively coupled plasma mass spectrometry (PEAA700, Switzerland) was used to determine the concentration of metal ions in digestion liquid, with the operations and instrument settings being described in Text S6 (Supporting Information).

Statistical analysis. The analysis of variance was used to assess the significance of experimental results. $p < 0.05$ was indicated statistically significant, while $p>0.05$ was considered statistically insignificant.

Results and Discussion

Impact of Digestion Liquid Pretreatment on Biochemical Methane Production. Fig. 1 shows the

150 measured methane production during the entire biochemical methane production assay period (i.e. 35 days) in
151 all the tests. The digester with a reflux ratio of 0% was set as the control. In the digester without digestion
152 liquid, methane production did not increase significantly after 25 days, so the best digestion time was 25 days,
153 and the maximum cumulative methane yield at this time was 172.1 ± 8.8 mL/g VSS. Although the
154 cumulative methane production trends of all the digesters with digestion liquid were similar, it can be clearly
155 found that the reflux of digestion liquid greatly affected methane production. For example, when the reflux
156 ratios of digestion liquid were 5%, 10%, 15%, 20% and 25%, the maximal cumulative methane yield were
157 232.2 ± 7.6, 249.7 ± 5.4, 263.8 ± 9.2, 282.5 ± 14.1 and 243.5 ± 12.2 mL/g VSS, respectively. The results
158 proved that it was feasible to increase methane production through proper reflux of digestion liquid.
159 From the values of R^2 shown in Fig. 1, it can be illustrated the satisfactory fit of methane production to
160 the first-order kinetic model in all the scenarios. Compared with the control without digestion liquid reflux,
161 a higher biochemical methane potential (B_0) can be achieved at any digestion liquid reflux ratio in this test.
162 Specifically, when the reflux ratios were between 20% and 25%, the maximum increase of B_0 was 63% (from
163 187 to 304 mL/g VSS). The measured methane production in Fig. 1 also clearly reflected this trend.
164 However, the reflux of the digestion liquid caused the determined hydrolysis rate (k) to be suppressed, which
165 was reduced by approximately 21% (from 0.14 to 0.11 d^{-1}). This was contrary to the conclusion of previous
166 studies that pretreatment of FA prepared by chemical increased K (Wei et al., 2017). The reason of this
167 conflict may be attributed to the effect of substances other than ammonium in the digestion liquid on
168 anaerobic digestion, which would be discussed in the following text.
169 **Methane Production from the Reflux Digestion Liquid.** Since there are some biodegradable
170 organics remaining in the digestion liquid, these organics may be bio-converted into methane. It was found
171 that when the volume of digestion liquid served as the sole substrate increased from 30 to 150 mL, the

maximal methane production increased from 8.9 ± 0.6 to 35 ± 0.5 mL (Fig. 2). Assuming that the total COD in the digestion liquid was completely converted to methane, the theoretical methane yields from 30, 60, 90, 120 and 150 mL digestion liquid were 10, 20.1, 30.1, 40.2 and 50.2 mL, respectively (Fig. 2), which only accounted for 1% - 4.6% of the corresponding measured methane production in Fig. 1. This indicated that the main factor for digestion liquid enhancing methane production was not the extra COD in digestion liquid.

Mechanism of How Digestion Liquid Enhances Methane Production. As the final product of WAS anaerobic digestion, methane production is related to several biological processes. Therefore, it is necessary to clarify the influence of digestion liquid on these processes. Sludge disintegration provide substrates, such as proteins and carbohydrates, for anaerobes to produce methane. The data shown in Fig. 3a indicated that the presence of digestion liquid promoted the disintegration of sludge. For example, when the reflux ratio of digestion liquid increased from 0 to 25%, the soluble COD (proteins) concentration after pretreatment was 1654.0 ± 51.0 mg/L (472.0 ± 22.3 mg/L) in the digester in the control (i.e., without digestion liquid), whereas the corresponding datum was 2453.7 ± 81.8 mg/L (1076.0 ± 24.7 mg/L) in the digester with 25% reflux ratio. Similar observations were also made at other reflux ratios. Fig. 3b shows EEM spectra of sludge liquid after 24 h alkaline pretreatment, which was often used to characterize the biodegradability of the released organics. Two main peaks (i.e., Peak A and Peak B) were found in the fluorescence spectra. Peak A and Peak B detected at excitation/emission wavelengths of 275/330-335 nm and 220/325-330 nm, were considered to aromatic proteins and tryptophan protein-like substances, respectively (Baker and Andy 2001). Compared with the control, refluxing digestion liquid caused a red shift of the emission wavelength and enhanced the fluorescence intensity (Fig. 3b), which once again suggested that refluxing digestion liquid promoted WAS to release more soluble substrates (Sheng and Yu, 2006).

It was observed from Fig. 3c that the refluxed digestion liquid inhibited rather than promoted the

bio-processes related to anaerobic digestion. For example, the specific degradation rates (mg/g VSS·h) in the control without refluxed digestion liquid were 28.9 ± 1.3 in BSA, 23.1 ± 1.2 in glucose, 20.0 ± 0.7 in propionate, 0.34 ± 0.02 in hydrogen and 30.6 ± 1.5 in acetate, whereas these values decreased to 23.0 ± 0.8, 22.6 ± 0.8, 19.0 ± 1.1, 0.13 ± 0.01 and 14.1 ± 0.7 with 20% reflux ratio of digestion liquid, respectively. The results suggested that refluxing digestion liquid had no effect on acidogenesis and acetogenesis but inhibited hydrolysis and methanogenesis. Moreover, methanogenesis was inhibited severer by digestion liquor than other processes.

Effect of Major Components in the Digestion Liquid on Methane Production. Digestion liquid includes several complicated substances, and their characteristics are quite different. These components were divided into five categories (i.e., ammonium, biodegradable organics, refractory organics, metal ions and anaerobes), and their impacts on methane production were further discussed.

There have been sufficient efforts to prove that FA improved the performance of anaerobic digestion (Wei et al., 2017; Wang et al., 2018). In the ammonium digester, the level of FA (~131 mg NH_3-N/L) produced from the added ammonium-synthetic medium increased methane production by 19% (Figure 4a). As expected, the ammonium in the digestion liquid was a beneficial element to improve WAS anaerobic digestion. Further exploration revealed that the main reason for this was that FA improved WAS solubilization rather than other biological processes (Fig. 4b and Table 2). All the results were well supported from previous report (Wang et al., 2018).

It can be seen from Fig.4 that compared with the control, the soluble COD and methane production in the biodegradable organics digester increased from 1654 ± 82.7 to 1794 ± 90.2 mg/L and 177.2 ± 8.9 to 183.6 ± 12.3 mL/g VSS, respectively. However, further analysis showed that these increases were similar to the theoretical conversion of organics in the added synthetic medium. In other words, the increment of SCOD

and methane production in the reactor containing biodegradable was likely to be the reconversion biodegradable organics in the added synthetic medium (Table S2). The phenomena outlined in Table 2 also support this speculation. The degradation rate of any model substrate in the biodegradable organics reactor was not significantly different ($p>0.05$) from the control. The results demonstrated the biodegradable organics in the digestion liquid will not affect all the processes of WAS anaerobic digestion.

From Fig. 4a, it can be seen an interesting observation that the metal ions in the digestion liquid obviously enhanced rather than inhibited the methane production. The presence of the metal ions significantly facilitated the biological processes related to anaerobic digestion, especially acetoclastic methanogenesis (Fig. 4b and Table 2). For instance, the degradation rate of the acetoclastic methanogenesis substrate in the metal ion digester was 54.37 ± 3.26 mg/(g VSS·d), which was 36.2% higher than that in the control (39.93 ± 4.76 mg/(g VSS·d)).

It was known that metal ions usually instruct the expression of enzymes, and the presence of metal ions at appropriate amounts may stimulate enzyme activities. For example, the methyl-CoM reductase containing nickel produced by strictly anaerobic archaea (e.g., methanogens) is one key enzyme that catalyzes the exergonic conversion of methyl-CoM and coenzyme B into methane and the heterodisulfide of coenzyme B and coenzyme M in final reaction of methanogenesis pathway (Ermler et al., 1997; Facchin et al., 2013). There are three subunits (i.e., α, β and γ,) in Methyl-coenzyme M reductase, which arrange as hexamer of $(\alpha\beta\gamma)_2$ composition. This multisubunit complex contains two cofactor F_{430} as a prosthetic group, which is responsible for methylation and demethylation in the catalytic cycle in methanogenesis, and must be activated with Ni as the centrally coordinated ion (Fig. 5a) (Livingston et al., 1984, Pfaltz et al., 1982). In addition, due to its plasticity in coordination and redox chemistry, Ni is often involved in enzymes related to the use and/or production of gases, such as carbon monoxide dehydrogenase, Acyl-coenzyme A synthetase, and nickel

238 acireductone dioxygenase (Ragsdale 2009).

239 On the contrary, the methane yield in the refractory organics reactor decreased. The methane yield from
240 the refractory organics reactor reduced from 177.2 ± 8.9 to 131.1 ±9.6 mL/g VSS, resulting in a reduction of
241 26% (Fig. 4a). Further analyses showed that the refractory organics in the digestion liquid did not promote
242 the decomposition of WAS (Figure 4b), but significantly restrained biological processes, especially
243 methanogenesis (Table 2). For example, the degradation rates of hydrogen and acetate in the reactor with
244 refractory organics were 5.68 ± 1.20 and 15.26 ± 1.01 mg/(g VSS·d), while these degradation rates in the
245 control were 12.65 ± 2.75 and 39.93 ± 4.76 mg/(g VSS·d). Based on the previous analysis method (Wang et
246 al., 2019), it can be concluded that this level of refractory organics inhibited the activities of microorganisms
247 related to hydrogentrophic methanogenesis and acetoclastic methanogenesis by 55.1% and 61.8%,
248 respectively.

249 According to the literature, some special functional microbes (e.g., microbes responsible for humic acid
250 reduction) compete with methanogens for the available substrates in anaerobic digestion processes.
251 Specifically, as shown in the Fig. 5b, the C-C and C-S bonds of acetyl CoA are cleaved by the carbon
252 monoxide dehydrogenase/acetyl-CoA synthase complex, which can also oxidize the carbonyl group with
253 transfer two electrons to the small protein electron carrier ferredoxin in the biotransformation of acetyl-CoA to
254 5-methyl-THMPT of acetoclastic methanogenesis process (Ferry 2010; Fischer and Thauer 1990). However,
255 the quinone groups of humic acid, which may come from the lignin or microbial metabolites, can serve as a
256 terminal electron-accepting moiety. This can encourage humic acid to compete for electrons, thereby
257 hindering ferredoxin regeneration (Liu et al., 2015; Cervantes et al., 2000), which may be the reason for the
258 low methane production in the refractory organics digester.

259 In the test to assess the effect of anaerobes in digestion liquid on methane production, the digestion liquid

260 treated with filter is considered sterile. Compared with the digestion liquid digester, the methane production
261 of the reactor containing the filtered digestion liquid decreased by 30.1%. It is easy to understand that the
262 refluxing digestion liquid will increase the abundance of microorganisms in the digestion system, so the
263 anaerobes involved in the digestion liquid were another contributor to promote methane production.

264 **Implications.** FA-based technology is increasingly recognized economically and practically for achieving
265 WAS reduction and energy recovery with the lowest cost through promoting a "closed-loop" concept in
266 WWTPs". In this conception, FA is regarded as a free chemical, which produced in suit from the waste (i.e.,
267 digestion liquid) of WWTPs (Fig. 6). All the previous achievements regarding FA, however, were based on
268 chemical (e.g., NH_4Cl) rather than real digestion liquid, possibly because this was a cutting-edge approach.
269 This means that the practical application of the FA-based technology was fraught with full indetermination
270 due to the complex composition of real digestion liquid. This study was the first attempt to promote the
271 anaerobic digestion of WAS by using actual digestion liquid as a source of FA, accelerating the FA-based
272 technology being adopted in real-world applications.

273 The results showed that both sludge reduction and methane production in the digester can be achieved in
274 a more economical way with refluxing the digestion liquid to the pretreatment unit. Based on the results
275 obtained in this work, it is calculated that this strategy can save ~$ 0.3 million cost in sludge transport and
276 disposal, and get ~$ 0.8 million benefit in energy production from methane annually in a WWTP
277 ($Q = 10^5$ m^3/day), as compared with the alkaline (pH 9.5, i.e., 0% reflux ratio in this work) pretreatment
278 method only (Table S3). Particularly, it was demonstrated that refractory organics in digestion liquid (e.g.,
279 humic acid) would severely suppress anaerobic digestion, and these organics could not be transformed or
280 removed in traditional digestion processes. In order to achieve a higher methane production, some extra
281 methods should be taken to eliminate the inhibition of refractory organics before anaerobic digestion. For

example, calcium peroxide can achieve a significant reduction in the contents of refractory organics, and perform well in promoting WAS solubilization (Wang et al., 2019).

The surprising discovery obtained in this work was that the metal ions in the digestion liquid had great potential for increasing methane production (Table 2), which may be related to the expression of key enzyme activities (Fig. 5a). The discovery of this phenomenon alleviates the scruple that the metal ions in the actual digestion liquid are harmful to anaerobic digestion. However, whether this conclusion obtained from the batch test had broad applicability needs to be fully verified by running long-term reactors in the future. Moreover, the main aim of this work is to assess the feasibility of refluxing digestion liquid to promote methane production and to reveal the underlying mechanism, therefore, parameters such as reflux ratios, pretreatment time, pH, inoculation method are not optimized. In addition, although the influence of single factors in the digestion liquid on WAS anaerobic digestion and their related mechanisms have been discussed in this work, the synergistic effect between two or three factors, such as metal ions, ammonium, anaerobes, biodegradable organics, recalcitrant organics was not investigated here. Thus, mathematical model and response surface method need to be used in the future to explore the synergistic effect among these various components in the digestion liquid before this strategy being implemented in full-scale applications.

Conclusion

The purpose of this work was to evaluate whether the refluxed digestion liquid can promote methane production from anaerobic digestion of WAS. The main conclusions are:

(1) As the reflux ratio of digestion liquid increased from 0% to 20%, the maximum cumulative methane production from anaerobic digestion of alkaline pretreated sludge increased from 174.2 ± 7.3 to 282.5 ± 14.1 mL/g VSS.

(2) Refluxing digestion liquid reduced the microbial activity associated with anaerobic digestion, but it

promoted WAS disintegration and improved the biodegradability of the released organics.

(3) Ammonium in the digestion liquid was the main booster to enhance methane production in the form of FA.

(4) It was found that the presence of metal ions (e.g., Ni) was beneficial to bio-processes of anaerobic digestion, which may be related to the activation of the key enzymes, such as methyl-coenzyme M reductase, and CO dehydrogenase.

(5) The refractory organics in digestion liquid were detrimental to anaerobic digestion through suppressing the bioprocesses, especially methanogenesis.

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Supporting Information

This file contains additional analytical methods Text S1-S6 and Table S1-S3.

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Table 1 Main characteristics of the WAS, inocula and digestion liquid

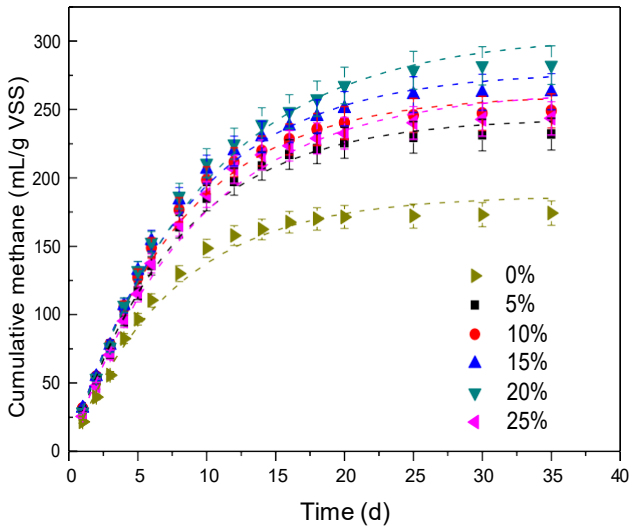
Parameters	WAS	Inocula	Digestion liquid
pH	6.9 ± 0.1	-	9.5±0.5
total suspended soils (TSS, mg/L)	22590±129	45660±680	-
VSS (mg/L)	9880± 87	39790±130	-
Total chemical oxygen demand (TCOD,mg/L)	12450 ± 170	48900 ± 430	956 ± 35
total carbohydrates (mg/L)	1060±67	-	30±7
total proteins (mg/L)	42700±157	-	107±33
NH_4^+-N (mg/L)	28±4	-	539±76
Short-chain fatty acids (SCFA, mg/L)	-	-	Acetic:301±88; Propionic:109±36
Humus (mg/L)	-	-	humic acid: 32±11; fulvic acid: 28±8
Lignocellulose (mg/L)	-	-	Hemicellulose:7.7±3;Lignin: 27±13;Cellulose:23±10;
Mental ions (mg/L)	-	-	Ni:2.2±0.5;Zn:3.5±0.8; Mn:0.61±0.05;Cu:0.15±0.03

Table 2 Effect of main abiotic components in the digestion liquid on the specific degradation rates of BSA, glucose, propionate, hydrogen, and acetate [a]

	Control	Biodegradable organics	Refractory organics	Ammonium	Metal ions
BSA	186.76 ± 8.14	193.92 ± 11.23	149.41 ± 7.62	183.97 ± 11.23	217.43 ± 12.72
Glucose	102.45 ± 5.86	105.33 ± 7.64	85.47 ± 7.02	99.73 ± 7.53	121.48 ± 6.82
Propionate	25.68 ± 3.78	22.87 ± 2.21	17.92 ± 2.31	23.53 ± 3.76	37.37 ± 2.56
Hydrogen	12.65 ± 2.75	11.26 ± 1.36	5.68 ± 1.20	7.07 ± 0.73	21.59 ± 1.08
Acetate	39.93 ± 4.76	35.87 ± 3.65	15.26 ± 1.01	19.56 ± 1.78	54.37 ± 3.26

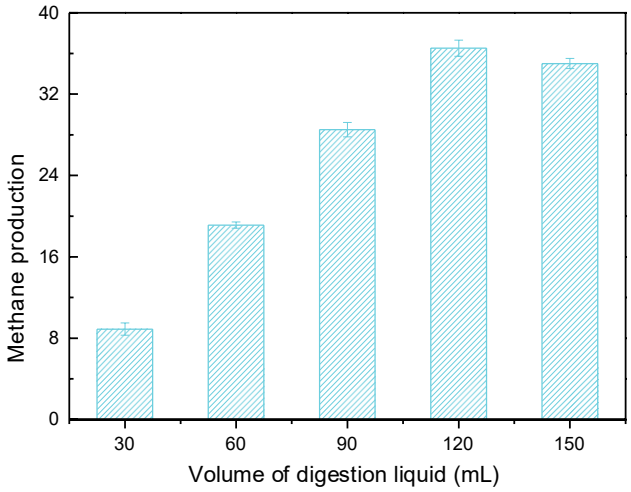
[a] Results are the averages and their standard deviations of triplicate tests, and the unit is mg/(g VSS·d).

Figure



Test	Parameters		R^2
	B_0 (mL/g VSS)	k (d^{-1})	
0%	187 ± 5.98	0.14 ± 0.004	0.9907
5%	243 ± 5.25	0.13 ± 0.005	0.9962
10%	260 ± 4.96	0.13 ± 0.004	0.996
15%	278 ± 6.93	0.12 ± 0.005	0.9948
20%	304 ± 8.59	0.11 ± 0.005	0.9948
25%	264 ± 7.81	0.11 ± 0.005	0.9939

Fig. 1 Measured and simulated methane production (using first-order kinetic model) from anaerobic digestion of WAS with different reflux ratios of digestion liquid . Symbols represent experimental data and lines represent model fit. Error bars represent standard deviations of triplicate tests. The data reported are net values, with the methane production from the inocula having already been subtracted.



Volume of digestion liquid (mL)	Total COD in digestion liquid (mg)	Theoretical CH_4 production (mL)
30	28.7	10
60	57.4	20.1
90	86	30.1
120	114.7	40.2
150	143.4	50.2

Fig. 2 The experimental and theoretical methane yield from different volumes of digestion liquid. The volumes of digestion liquid (i.e., 30, 60, 90, 120, and 150 mL) were equal to those being added into the 5%, 10%, 15%, 20%, and 25% reactors, respectively. Theoretical methane production referred to the value that the total COD in the digestion liquid was completely converted to methane, and the calculated equation: $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$. Error bars represent standard deviations of triplicate tests.

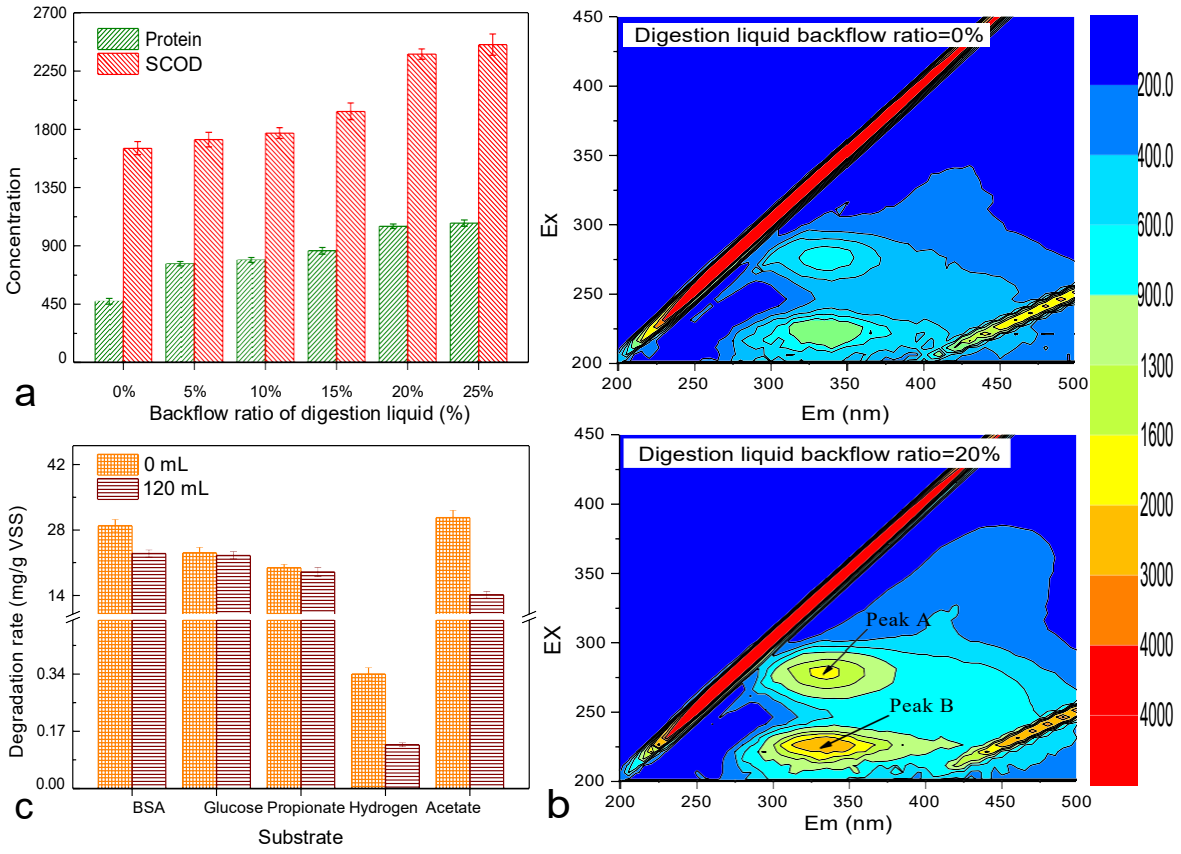
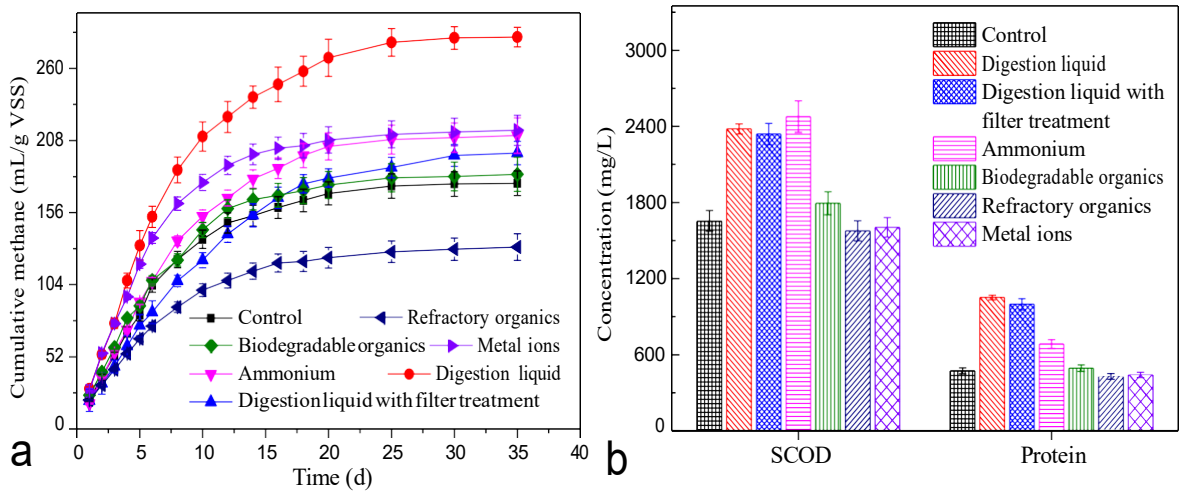


Fig. 3 Soluble COD and proteins (a) and EEM profiles of liquid (b) after 24 h alkaline pretreatment, and the effect of digestion liquid on the specific degradation rates of BSA, glucose, propionate, hydrogen, and acetate (c). Error bars represent standard deviations of triplicate tests.



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21 **Fig. 4 Effects of main components of digestion liquid on cumulative methane production (a) and soluble**

22 **COD and proteins after alkaline (pH 9.5) pretreatment for 24 h (b). Error bars represent standard**

23 **deviations of triplicate tests.**

24

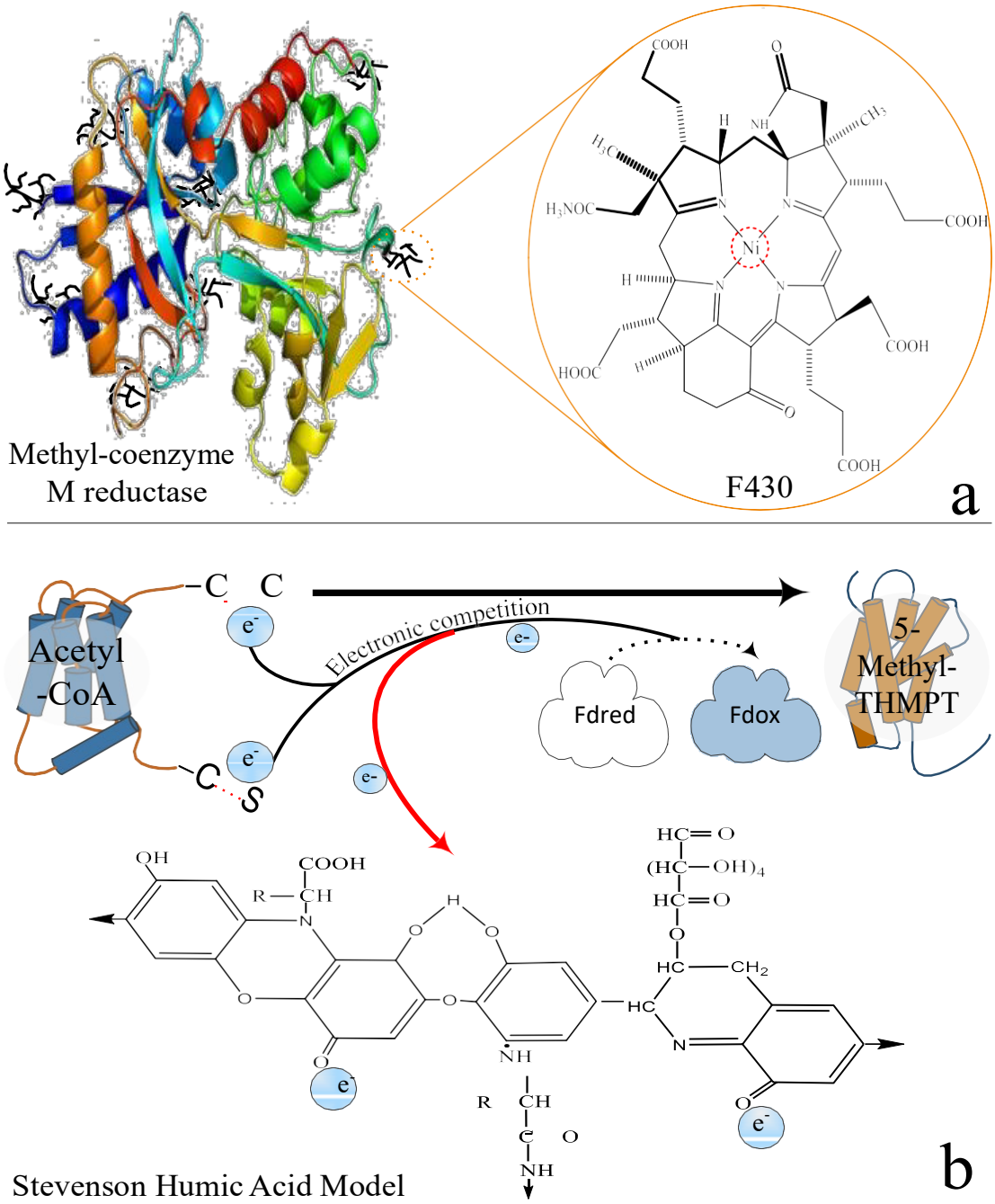
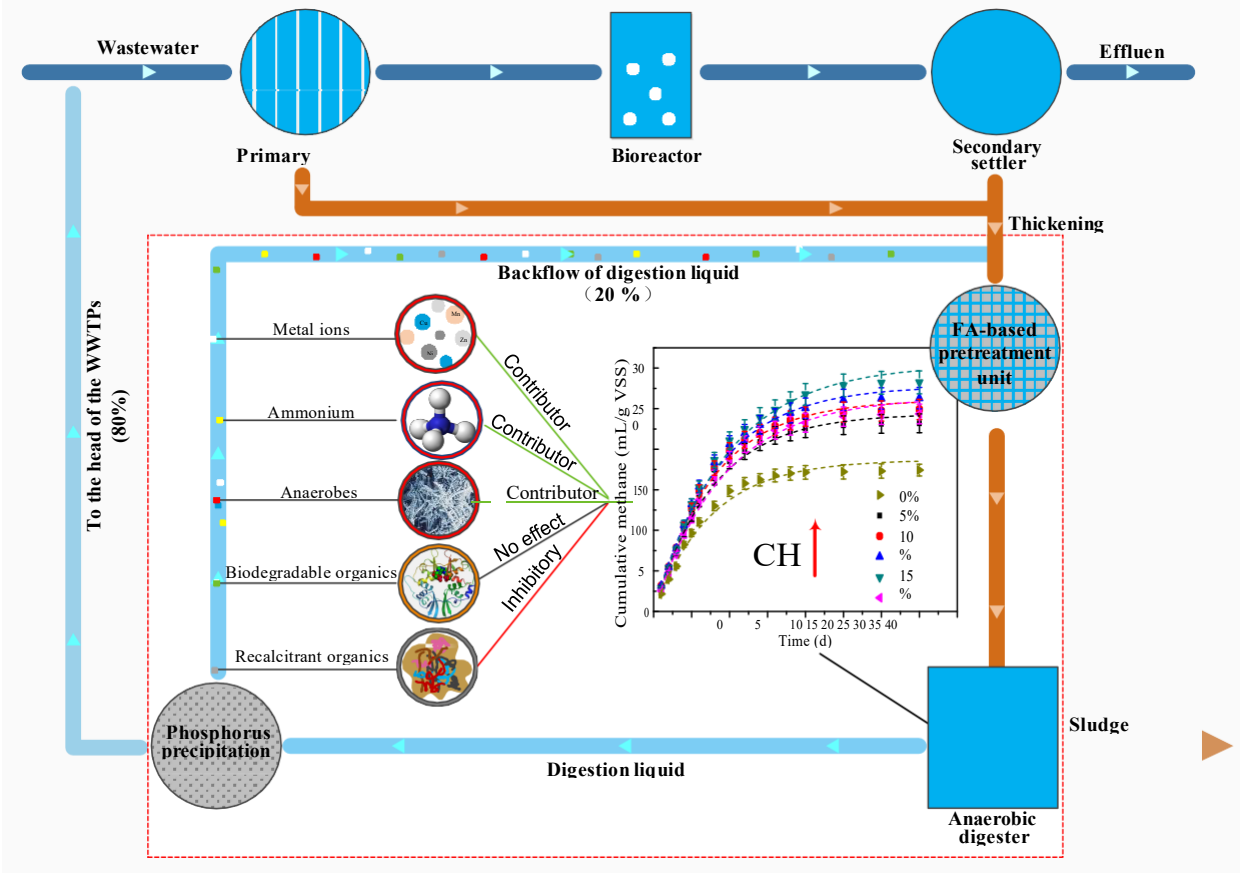


Fig. 5 Schematic diagram of how metal ions (a) and humic acid (b) affect the activity of key enzymes and methane production according to the reference (Liu et al., 2015; Ermler et al., 1997; Ferry, 2010). Subunits α(blue), α' (orange), γ (red), β' (green), β (light blue), γ' (yellow) and coenzyme F430 (black); THMPT: tetrahydromethanopterin; Fdox: oxidized ferredoxin; Fdred: reduced ferredoxin.



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32 **Fig. 6 Major findings in this work and a "closed-loop" concept in a WWTP using digestion liquid to**

33 **increase methane production.**

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