

1 Influence of feed/inoculum ratios and waste cooking oil content on the  
2 mesophilic anaerobic digestion of food waste

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8 **Abstract**

9 Information on the anaerobic digestion (AD) of food waste (FW) with different waste  
10 cooking oil contents is limited in terms of the effect of the initial substrate  
11 concentrations. In this work, batch tests were performed to evaluate the combined  
12 effects of waste cooking oil content (33–53%) and feed/inoculum (F/I) ratios (0.5–1.2)  
13 on biogas/methane yield, process stability parameters and organics reduction during  
14 the FW AD. Both waste cooking oil and the inoculation ratios were found to affect  
15 digestion parameters during the AD process start-up and the F/I ratio was the  
16 predominant factor affecting AD after the start-up phase. The possible inhibition due  
17 to acidification caused by volatile fatty acids accumulation, low pH values and  
18 long-chain fatty acids was reversible. The characteristics of the final digestate  
19 indicated a stable anaerobic system, whereas samples with F/I ratios ranging from 0.8  
20 – 1.2 display higher propionic and valeric acid contents and high amounts of total  
21 ammonia nitrogen and free ammonia nitrogen. Overall, F/I ratios higher than 0.70  
22 caused inhibition and resulted in low biogas/methane yields from the FW.

23 **Keywords**

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24 Anaerobic digestion of food waste; Biogas; Waste cooking oil; Feed/inoculum ratios;

25 Digestate

26 **Abbreviations**

27 FW: Food Waste

28 AD: Anaerobic Digestion

29 F/I: Feed/Inoculum

30 EE: Ether Extract

31 VFA: Volatile Fatty Acids

32 LCFA: Long Chain Fatty Acids

33 SFA: Saturated Fatty Acids

34 MUFA: Monounsaturated Fatty Acids

35 PUFA: Polyunsaturated Fatty Acids

36 RT: Retention Time

37 TS: Total Solid

38 VS: Volatile Solid

39 AMPTS: Automatic Methane Potential Test System

40 TAN: Total Ammonia Nitrogen

41 FAN: Free Ammonia Nitrogen

42

## 43 **1. Introduction**

44 Anaerobic digestion (AD) has been widely applied to reduce the volume of food  
45 waste (FW) and to recover energy (e.g., methane) from FW. The content of waste  
46 cooking oil in FW may vary from 1% to 5% (wet basis) (Li et al., 2016; Nie et al.,  
47 2013) due to different eating habits, cooking methods and local cultures (Koch et al.,  
48 2015). In addition, waste cooking oil often results in a higher biochemical methane  
49 production than carbohydrates and protein (Angelidaki and Sanders, 2004). However,  
50 the FW biodegradation processes can be hampered by long-chain fatty acids (LCFAs),  
51 which are produced from waste cooking oil and can cause toxicity to microorganisms  
52 and biomass adsorption (Chen et al., 2014).

53 Previous studies have reported various inhibitory concentrations of lipids, including  
54 31–47% for chemical oxygen demand basis (Cirne et al., 2007) and 65% for volatile  
55 solid (VS) basis (Sun et al., 2014). FW with lipid contents higher than 35% have been  
56 shown to result in AD processes with longer lag phases and lower first-order  
57 degradation constants (Zhang et al., 2017). Studies have also shown that the inhibition  
58 caused by LCFAs varies depending on the type of feedstock and is more correlated  
59 with the physical characteristics (e.g., specific surface area and size distribution) than  
60 the biological characteristics (e.g., inoculum origin, specific acetoclastic  
61 methanogenic activity and inoculum adaptation to lipids) of the process (Chen et al.,  
62 2008; Hwu et al., 1996). However, these studies were often carried out using either  
63 model lipid-rich waste (Cirne et al., 2007) or edible oil (Sun et al., 2014), which have  
64 significantly different characteristics from those of waste cooking oil in FW. The

65 waste cooking oil existed in FW is of low hygiene quality (Ren et al., 2013; Zhang et  
66 al., 2003) and has higher triacylglycerol content (e.g., C14:0, C16:1, C16:0 and  
67 C17:0), oleic acid (C18:1) and linoleic acid (C18:2) contents (Zhuang et al., 2013)  
68 that are present in the intermediates generated during AD and are considered to be the  
69 main inhibitory factors of LCFAs (Alves et al., 2009). Therefore, investigating the  
70 influence of the waste cooking oil ratio on FW digestion performance is necessary.

71 An excessive amount of biomass substrate may lead to the accumulation of total  
72 ammonia-nitrogen (TAN) and volatile fatty acids (VFA), resulting in an inhibitory  
73 effect on the biogas yield (Fernández et al., 2008; Zhao et al., 2017). Studies have  
74 shown that the inhibition caused by LCFAs can be alleviated by increasing the  
75 biomass/LCFA ratio using inoculums (Palatsi et al., 2009), and that methane  
76 production can decrease or even stop without proper F/I ratios. Additionally, F/I ratios  
77 have been reported to affect methane yield mainly with substrates derived from durian  
78 shells (Zhao et al., 2017), food and green wastes (Liu et al., 2009), swine slurries  
79 (González-Fernández and García-Encina, 2009), wheat straws, whole crop maize,  
80 cattle manure, grass, cellulose (Moset et al., 2015) and other organic wastes  
81 (Boulanger et al., 2012; Dechruga et al., 2013; Fagbohunbe et al., 2015; Haider et  
82 al., 2015; Pellerá and Gidarakos, 2016). However, considering the potential VFA  
83 production and the buffering capacity of the medium using ammonium, each substrate  
84 has its own optimum feed/inoculum (F/I) ratio (Lesteur et al., 2010). Moreover,  
85 studies examining the combined influence of waste cooking oil and F/I ratios on  
86 process stability and biogas/methane productivity in the AD of FW are still lacking. A

87 literature review of ether extract (EE) content in FW showed that EE accounts for  
88 approximately 6-45% of the total FW in China (VS basis) (Li et al., 2016; Nie et al.,  
89 2013). Since lipid-rich waste is more likely to result in operational problems (Chen et  
90 al., 2008; Cirne et al., 2007; Long et al., 2012), the influence of higher waste cooking  
91 oil ratios, specifically EE/VS feedstock ratios ranging from 33% to 53%, were  
92 investigated in the present study (Table 1).

93 This paper aims to investigate the AD characteristics of FW containing different  
94 waste cooking oil and F/I ratios. The modified Gompertz model was applied to  
95 describe biogas production process and to determine the digestion efficiency, which  
96 were then further evaluated to determine how and over which ranges the two ratios  
97 affect digestion performance, process kinetics and biodegradability. From this analysis,  
98 possible inhibitory effects were discussed, and the optimal waste cooking oil and F/I  
99 ratios that increased methane yields were presented.

## 100 **2. Materials and Methods**

### 101 *2.1. Substrates and inoculum*

#### 102 *2.1.1. FW*

103 FW was collected from a school canteen in Beijing, China. Impurities in the  
104 collected FW (e.g., big bones, plastics and metals) were manually removed before the  
105 FW was macerated into 1 – 2 mm particles. The main characteristics of the FW used  
106 in the experiments were (average values of three determinations with standard  
107 deviations) shown in Table 1.

108 Some samples were used to extract waste cooking oil with petroleum ether  
 109 (analytically pure, boiling point: 30–60 °C) using a rotary evaporator at 60 rpm. Then  
 110 the extracted oil was used for adjusting the waste cooking oil ratio in FW, and the  
 111 ratio was characterized by the concentration of the EE in the VS of the FW (EE/VS):

$$112 \quad EE/VS = \frac{m_{FW} \times EE_{FW} \% + m_{oil-extracted}}{m_{FW} \times VS \% + m_{oil-extracted}} \times 100\% \quad (1)$$

113 where  $m_{FW}$  is the mass of the initial FW,  $EE_{FW} \%$  is the percent of EE in the  
 114 initial FW sample,  $m_{oil-extracted}$  is the mass of waste oil added in the FW, which was  
 115 extracted from FW, and  $VS\%$  is the VS content of the initial FW. [Table 2](#) presents the  
 116 LCFA composition of the waste cooking oil in the FW.

117 **Table 1.** Characteristics of the FW and the F/I ratios.

Items	Waste cooking oil ratios (EE/VS)						
	33%	36%	40%	43%	46%	50%	53%
pH	4.47±0.21	4.46±0.32	4.46±0.28	4.46±0.41	4.45±0.32	4.45±0.29	4.44±0.44
TS <sup>a,b</sup> (%)	15.01±0.98	15.47±0.71	16.41±0.63	17.02±0.42	17.76±0.45	18.71±0.29	19.93±0.35
VS <sup>a,c</sup> (%)	14.18±0.52	15.52±0.82	18.06±0.91	19.71±0.71	21.63±0.50	23.98±0.46	26.90±0.49
Protein <sup>a</sup> (%)	3.58±0.15	3.56±0.22	3.53±0.06	2.17±0.06	2.15±0.33	2.12±0.17	2.09±0.28
F/I <sup>d</sup>	1.20	1.00	0.80	0.70	0.60	0.56	0.50

118 <sup>a</sup>: wet basis; <sup>b</sup>: total solid; <sup>c</sup>: volatile solid; <sup>d</sup>: feed to inoculum ratio

119 **Table 2.** LCFA composition of the waste cooking oil extracted from the FW (%).

SFA <sup>a</sup>	Content	Unsaturated fatty acid			
		MUFA <sup>b</sup>	Content	PUFA <sup>c</sup>	Content

		Myristoleic acid			
Lauric acid (C12:0)	0.05		0.04	Linoleic acid (C18:2n6)	26.46
		(C14:1)			
		Palmitoleic acid			
Myristic acid (C14:0)	0.88		1.49	$\gamma$ -Linolenic acid (C18:3n6)	0.09
		(C16:1)			
Palmitic acid (C16:0)	23.37	Oleic acid (C18:1)	33.55	$\alpha$ -Linolenic acid (C18:3n3)	1.82
		Gondoic acid			
Stearic acid (C18:0)	9.25		1.09	Eicosadienoic acid (C20:2)	0.21
		(C20:1)			
		Arachidic acid			
	0.43	Erucic acid (C22:1)	0.21	Arachidonic acid (C20:4n6)	0.38
		(C20:0)			
Behenic acid (C22:0)	0.06	Nervonic acid (C24:1)	0.10	Mead acid (C20:3)	0.03
		Lignoceric acid		Eicosapentaenoic acid	
	0.15				0.15
		(C24:0)		(C20:5n3)	
				Docosahexaenoic acid	
					0.19
				(C22:6n3)	
Total	34.19		36.48		29.33

120 <sup>a</sup> SFA: Saturated Fatty Acid;

121 <sup>b</sup> MUFA: Monounsaturated Fatty acid;

122 <sup>c</sup> PUFA: Polyunsaturated Fatty Acid.

### 123 2.1.2. Inoculum

124 Seed sludge was obtained as an inoculum from a steady-operation digester (37 °C)

125 at a wastewater treatment plant in Beijing, China. After a two-day gravity

126 sedimentation period, the supernatant was discarded, and the remainder was passed

127 through a 2-mm sieve to remove large particles/grit. The characteristics of the

128 inoculum are shown in [Table 3](#).

129 **Table 3.** Characteristics of the inoculum.

Parameter	pH	TS (%)	VS (%)	Ammonia (mg/L)	C/N <sup>a</sup>
Value	7.34	3.65%	2.42%	1123	7.01

130 <sup>a</sup>: carbon to nitrogen ratio

## 131 2.2. AD experimental setup

### 132 2.2.1. Determination of the inoculum ratios

133 To identify the synergistic impacts of the F/I and EE/VS ratios on FW digestion, we  
 134 focused on their interactions with inoculum ratios in digestion experiments on a VS  
 135 basis (Table 1). All the F/I ratios as shown in Table 1 were based on a mass of VS  
 136 basis.

### 137 2.2.2. Batch digestion tests

138 Batch tests were conducted in 15 parallel 500-mL glass bottles at 37 °C with an  
 139 automatic methane potential test system II (AMPTS II) that was supplied by  
 140 Bioprocess Control (Lund, Sweden). AMPTS II features automatic sample stirring, an  
 141 acid gas (such as CO<sub>2</sub> or H<sub>2</sub>S) removal system and a biomethane yield recording  
 142 system. The system performs fast and accurate on-line measurements of ultra-low  
 143 biogas and biomethane flow to determine the biogas potential. All the reactors were  
 144 started simultaneously and used synchronous agitation at the same speeds (160 r/min)  
 145 and intervals (60 seconds on/off).

146 The substrates and inoculums were placed into bottles with different F/I ratios. The  
 147 upper area of each reactor was flushed with nitrogen for at least 1 min to ensure  
 148 anaerobic conditions and was then quickly sealed. All of the reactors were placed in a



149 water bath to maintain the digestion system at a mesophilic temperature (37 °C) for  
150 AD. For each test, three samples were examined, and two digesters containing only  
151 inocula were incubated to correct for the biogas yield from the inoculum. The biogas  
152 yield was calculated by the VS of substrate in the bottle, including FW and waste  
153 cooking oil added. The digestion assays were stopped when the daily biogas (or  
154 methane) production was less than the 1% of the total accumulated biogas (or  
155 methane).

### 156 *2.2.3. Digesters with two volume types*

157 An AMPTS II system containing 500-mL (total volume) glass bottles (*A*) was used  
158 to measure real-time methane productivity and kinetics, whereas a system with 2-L  
159 (total volume) glass bottles (*B*) was used for sample collection and detection. All of  
160 the bottles in both systems were fed with the same samples and inoculums with  
161 different F/I ratios (Table 1). To achieve accurate results, collecting samples at the  
162 correct times (e.g., the inhibition stage, recovery stage and final stage) is important.  
163 Digestion system *A* was started two days prior to system *B* to understand how the  
164 sample collection time affected the methane yield patterns.

### 165 *2.3. Kinetics study*

166 A kinetics analysis can provide insights into the influences of the F/I and EE/VS  
167 ratios on the potential behaviour of organics degradation in the digestion system, such  
168 as the lag phase, which is an important factor in determining the AD efficiency. The  
169 modified Gompertz model was used to describe the biogas yield potential, the lag  
170 phase and the maximum biogas production rate:

171 
$$M = P \times \exp \left\{ -\exp \left[ R_{\max} \times e \times (\lambda - t) / P + 1 \right] \right\} \quad (2)$$

172 where  $M$  is the cumulative biogas production (mL/g VS) at the digestion time  $t$ ,  $P$  is  
173 the biogas production potential (mL/g VS),  $R_{\max}$  is the maximum biogas production  
174 rate (mL/g VS h),  $\lambda$  is the lag time (h),  $t$  is the retention time (h), and  $e$  is the  
175 exponential constant 2.7183.

## 176 2.4. Statistical analysis

### 177 2.4.1. Pearson correlation analysis

178 The Pearson correlation ( $p < 0.05$ ) was determined to describe significant  
179 relationships between the above parameters, using the IBM SPSS Statistics 20  
180 software (Table A1).

### 181 2.4.2. Second-order polynomial model analysis

182 Response surface methodology was used to describe the relationship between the  
183 responses and independent variables. The functional relationships between the  
184 responses ( $M$ ) and the set of factors ( $X$  and  $Y$ ) were described by estimating the  
185 coefficients of the following second-order polynomial model based on the  
186 experimental data:

187 
$$M = M_0 + aX + bY + cX^2 + dY^2 + fXY \quad (3)$$

188 where  $M$  is the predicted response,  $M_0$  is a constant,  $X$  and  $Y$  refer to the EE/VS and  
189 F/I ratios, respectively,  $a$  and  $b$  are linear coefficients,  $c$  and  $d$  are quadratic  
190 coefficients, and  $f$  is the interaction coefficient.

191 Additionally, the results and the coefficients of the quadratic equation were  
192 analysed using ANOVA ( $p < 0.05$ ) via the R software 3.3.2 package.

193 *2.5. Analytical methods*

194 The pH was measured using a pH metre (FE20, Mettler, Switzerland). The TS and  
195 VS were determined according to the standard methods of the American Public Health  
196 Association (APHA, 1992). VFAs were measured using an Agilent Gas  
197 Chromatograph (Agilent GC-7890A, California, USA) equipped with a flame  
198 ionization detector. The concentrations of protein and EE were determined with the  
199 Kjeldahl method and a Soxhlet device, respectively (Naumann and Bassler, 1993).  
200 The concentrations of TAN and free ammonia nitrogen (FAN) were determined as  
201 previously reported (Siles et al., 2010). LCFAs were determined in accordance with  
202 the method of (Palatsi et al., 2009).

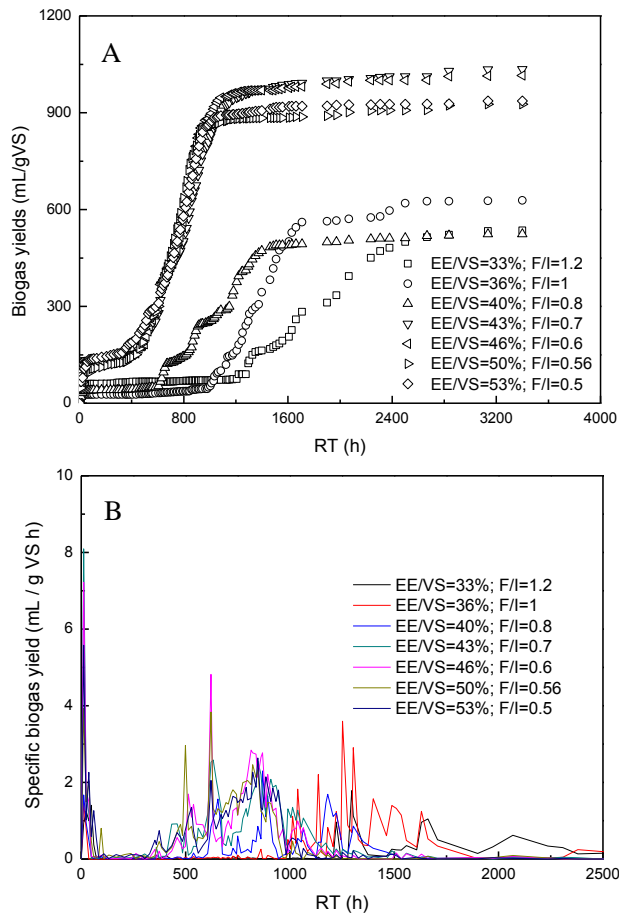
203 **3. Results and Discussion**

204 *3.1. Biogas production and methane content*

205 *3.1.1. Characteristics of biogas production and methane content*

206 (1) Cumulative biogas yield

207 The biogas yield ranged from 524 to 1035 mL/g VS after 150 days of digestion,  
208 whereas the methane percentage of the biogas varied between 67% and 75% (Fig. 1A).  
209 Lipids had the highest biochemical methane potential of the FW contents (Angelidaki  
210 and Sanders, 2004), therefore, FW with higher EE/VS and lower F/I ratios resulted in  
211 higher biogas yields ( $p<0.05$ ) and higher methane contents ( $p<0.01$ ). The highest  
212 yield was achieved from FW with an EE/VS ratio of 43% and an F/I ratio of 0.70 and  
213 FW with an EE/VS ratio of 46% and a F/I ratio of 0.60, ranging from 1015 to 1035  
214 mL/g VS. These two treatments were 8.44–93.70% higher than the other treatments.



215

216 **Fig. 1.** Effect of EE/VS and F/I ratios on the cumulative (A\*) and specific (B) biogas  
 217 yield with increased retention time (RT).

218 \*: average biogas yields.

219 (2) Specific biogas yield rate

220 The degradation of the FW exhibited intense production during the first 30–40 h,  
 221 followed by broader and smaller peaks (Fig. 1B). In the sample with F/I and EE/VS  
 222 ratios of 0.7 and 43%, respectively, relatively intense methane production occurred,  
 223 with the peak value achieved within the first 12 h (8 mL/g VS h).

224 Two main peaks were obtained from the samples. The first peak was of shorter  
 225 duration and larger maximum height than the second peak. The occurrence of these

226 peaks was due to the degradation of easily degradable organics and macromolecular  
227 insoluble materials (such as proteins and lipids). The different peak patterns likely  
228 result from different FW organic compositions, particularly lipid ratios (EE/VS). The  
229 first peak may be due to the biodegradation of carbohydrates, which are converted  
230 more rapidly than lipids and proteins (Mata-Alvarez et al., 2000). The second peak  
231 may be attributed to the combined degradation of proteins and lipids as well as any  
232 remaining carbohydrate.

233 Table 4 summarizes the biogas yield, starting/ending times and durations of these  
234 two peaks. Lower biogas/biomethane yields and pH values (ranging from 5.14 to 5.55)  
235 were achieved at the end of the first stage, and the second stage was characterized by  
236 higher biogas production (408–786 mL/g VS) and a longer retention time (767–1465  
237 h). Biogas was mainly produced in the second stage, which accounted for 73–83% of  
238 the total production, and the retention time for the second stage was 1.35–3.97 times  
239 as long as that of the first stage. The lower biogas yield in the first stage was probably  
240 influenced by decreased pH, which is not compatible with normal methanogens  
241 growth (Zhang et al., 2014). Moreover, higher F/I and lower EE/VS ratios resulted in  
242 longer retention times for the two stages, and a significant correlation between these  
243 parameters ( $p < 0.01$ ) was achieved, while only the F/I ratio significantly correlated  
244 with the biogas yield in the second stage (Table A1). These findings are likely due to  
245 both the F/I and EE/VS ratios being the main factors that affect AD start-up (first  
246 stage), which is associated with the initial production of fermentative products. After  
247 the start-up phase (second stage), the F/I ratios became the predominant factor

248 affecting the bio-transform of initial fermentation products by the acetogenic and  
 249 methanogenic communities.

250 **Table 4.** Characteristics of biogas production from the FW.

EE/VS	33%	36%	40%	43%	46%	50%	53%
F/I	1.20	1.00	0.80	0.70	0.60	0.56	0.50
<b>1. The first stage of biogas production</b>							
Starting time (h)	0	0	0	0	0	0	0
Ending time (h)	1085	967	552	304	407	360	262
Duration (h)	1085	967	552	304	407	360	262
Biogas yield (mL/g VS)	72	44	49	145	148	129	151
Percentage in total <sup>a</sup> (%)	13	7	9	14	15	14	16
pH at the ending time	5.48	5.55	5.14	5.24	5.47	5.46	5.55
<b>2. The second stage of biogas production</b>							
Starting time (h)	1085	967	552	304	407	360	262
Ending time (t <sub>90</sub> ) <sup>b</sup> (h)	2550	2308	1708	1510	1222	1173	1029
Duration (h)	1465	1341	1156	1206	815	813	767
Biogas yield (mL/g VS)	408	520	419	786	765	704	686
Percentage in total <sup>a</sup> (%)	76	83	80	76	75	76	73
pH at the ending time	7.11	7.14	6.99	7.25	7.12	7.20	7.22
<b>3. Total AD process</b>							
V <sub>90</sub> <sup>c</sup> (mL/g VS)	480	564	468	931	912	833	837
Total biogas yield (mL/g VS)	535	628	524	1035	1015	926	936
Methane content (%)	67	67	72	73	72	74	75
TBY <sup>d</sup> (mL/g VS)	1129	1130	1133	1098	1110	1128	1140
TMBY <sup>e</sup> /TBY (%)	47	56	46	94	91	82	82

251 <sup>a</sup> Ratios of the biogas yield in this stage to total biogas yield in the whole digestion process.

252 <sup>b</sup> t<sub>90</sub>: Time taken for 90% biogas production.

253 <sup>c</sup> V<sub>90</sub>: Volume of 90% of total biogas production (90% × total biogas production).

254 <sup>d</sup> Theoretical biogas yield (*TBY*) was calculated according to the reference (Labatut et al., 2011).

255 <sup>e</sup> *TMBY*: Total measured biogas yield.

256 The ratios of measured biogas yield to theoretical biogas yield varied from 47% to  
257 94%, indicating a lower biogas conversion efficiency for higher F/I ratios. The highest  
258 yield ratio (94%) was achieved with F/I and EE/VS ratios of 0.70 and 43%,  
259 respectively, whereas the highest biogas yield was achieved with a methane  
260 proportion of 73% in the biogas (Table 4). These results indicated that the F/I and  
261 EE/VS ratios are essential factors that influence the biogas yield, methane content and  
262 digestion time during the batch AD of FW.

### 263 3.1.2. Kinetics evaluation

264 Table 5 summarizes the biogas production potential (*P*), maximum biogas yield  
265 rate ( $R_m$ ) and lag time ( $\lambda$ ) according to a modified Gompertz model. The high  
266 determination coefficients ( $R^2$ , 0.9173–0.9822) and high *P* to total measured biogas  
267 yield ratios (94–103%) for all of the runs showed that the experimental biogas  
268 production data could be well simulated using this model.

269 **Table 5.** Results of the kinetics parameters from the modified Gompertz model.

EE/VS	F/I	$P$ (mL/g VS)	$R_m$ (mL/g VS h)	$\lambda$ (h)	$R^2$	$P/TMBY^a$ (%)	$P/TBY^b$ (%)	$\lambda/t_1^c$ (%)
33%	1.20	517	0.29	872	0.9173	103	46	80
36%	1.00	615	0.79	963	0.9822	102	54	100
40%	0.80	537	0.61	561	0.9796	98	47	102
43%	0.70	1063	1.19	267	0.9644	97	97	88
46%	0.60	1075	1.46	347	0.9633	94	97	85
50%	0.56	905	2.07	430	0.9760	102	80	119
53%	0.50	953	1.52	342	0.9611	98	84	131

270 <sup>a</sup> *TMBY*: Total measured biogas yield.

271 <sup>b</sup> *TBY*: Theoretical biogas yield (*TBY*) was calculated according to the reference (Labatut et al., 2011).

272 <sup>c</sup>  $t_1$ : Ending time of the first stage of biogas production in Table 4.

273 Samples with lower EE/VS ( $p < 0.05$ ) and higher F/I ratios ( $p < 0.01$ ) had longer  
 274 lag times, especially samples with F/I ratios higher than 0.80 (ranging from 561 to  
 275 963 h), even if the waste cooking oil content was relatively low (33–40%). These  
 276 findings may be due to diffusion limitations imposed by the lipid layer surrounding  
 277 the bacterial cells at high organic loadings, the slow degradation of lipids or the  
 278 possible inhibition of methanogenic activity by high LCFA concentration (Chen et al.,  
 279 2014; Long et al., 2012). The F/I ratio could drive the start-up phase of the anaerobic  
 280 digester, likely due to the degradation of initial hydrolysis products by the  
 281 methanogenic consortia. Additionally, decreasing F/I ratios may dilute the inhibitory  
 282 or toxic compounds produced from LCFAs, which was also confirmed by the longer



283 duration of the first stage of biogas production (Table 4).

284 Samples at EE/VS and F/I ratios of 43% and 0.70, respectively, had the shortest lag  
285 times and achieved high biogas production potential (1063 mL/g VS). Samples with  
286 lower EE/VS and higher F/I ratios exhibited lower biogas yields and lower ratios of  $P$   
287 to total measured biogas yield (Table 5). These low values may be due to acidification  
288 during the first digestion stage. Moreover, further increases in the EE/VS ratio and  
289 decreases in the F/I ratio resulted in longer lag times and lower biogas production.  
290 Thus, feedstock with higher EE/VS ratios (46–53%) requires lower F/I ratios than  
291 those currently used to minimize and overcome inhibition.

292 The  $\lambda$  values exhibited significant positive correlations ( $p < 0.01$ ) with the  $t_{90}$  values  
293 (Table A1), and assays with a longer  $\lambda$  values had lower biogas yields and methane  
294 content. Additionally, samples with high F/I ratios had significantly ( $p < 0.01$ )  
295 decreased  $R_m$  values (0.29–2.07 mL / (g VS h)) due to their long lag phases, whereas  
296 the EE/VS ratios exhibited positive effects ( $p < 0.01$ ). Therefore, a high F/I ratio  
297 increases the duration of the adaptation phase. This result indicated that a good F/I  
298 ratio (i.e., less than 0.7) is beneficial for microorganism growth and biogas production,  
299 and high inoculum concentrations may shorten the digestion time.

300 In addition, higher  $R_m$  values resulted in shorter lag phases, lower  $t_{90}$  values, and  
301 higher biogas production. High methane conversion efficiency for EE compared with  
302 carbohydrates and proteins may lead to high biogas yield rates, implying that a  
303 relatively large EE/VS ratio is good for the AD system. The  $R_m$  values have a positive  
304 correlation ( $p < 0.05$ ) with the  $P$  values (517–1075 mL/g VS), whereas the  $\lambda$  values

305 exhibited negative effects ( $p < 0.05$ ). These relationships likely indicate that high  
306 biogas conversion efficiency and quick adaptation to a new substrate are essential  
307 factors for an inoculum that influence the ultimate biogas yield in the batch AD of  
308 FW.

### 309 *3.2. Characteristics of the performance parameters and possible inhibition*

#### 310 *3.2.1. VFAs*

311 As shown in [Table 4](#), various durations of the first stage of biogas production were  
312 achieved due to the differences in the EE/VS and F/I ratios. Since the VFA  
313 concentration had a significant influence on the pH value, it is essential to study  
314 variations of the VFA concentration and composition, especially in the first stage from  
315 day 11 to 45 ([Fig. 2](#)).

316 The VFA concentration first increased continuously to a peak, and assays with  
317 lower EE/VS and relatively higher F/I ratios presented higher peak values, indicating  
318 a rapid build-up of VFAs. Because of their high F/I ratios, the quantities of  
319 microorganisms were too low to degrade the initial fermentation products in the  
320 soluble fraction of these samples. Therefore, more time was needed to decrease their  
321 VFA concentrations, corresponding to longer lag phases. Additionally, during the first  
322 stage of biogas production, the low pH values (ranging from 5.1 to 5.6 at the end of  
323 the first biogas yield stage) caused by the high VFA concentrations resulted in  
324 inhibition of the methane yield process ([Table 4](#)), indicating low biogas yields as  
325 retention time increased until the end of the first biogas yield stage ([Fig. 1A](#)).

326 However, after an initial lag phase (267–963 h), the accumulated VFA concentration

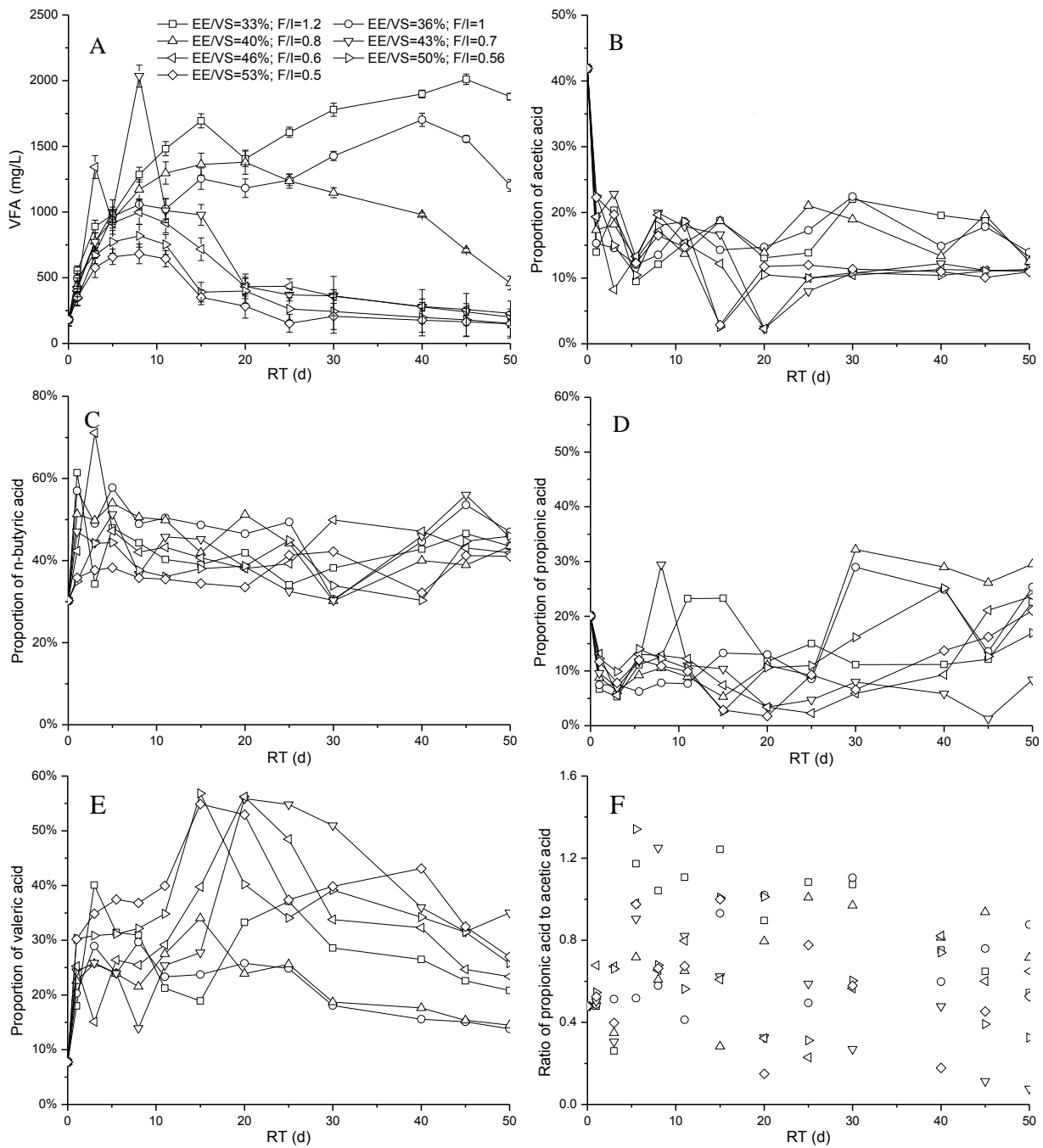
327 decreased, resulting in a pH increase, which caused the biogas production process to  
328 start again. These findings imply that the acidification caused by high VFA  
329 concentrations and consequent low pH values in the present study are reversible. This  
330 hypothesis was strengthened by the fact that acetogenic and methanogenic  
331 communities are much more sensitive to low growth rates compared with  
332 fermentative and hydrolytic populations (Niu et al., 2014).

333 Acetic acid and *n*-butyric acid are the two main components of the total VFAs,  
334 accounting for 50–72% (Fig. 2). Samples with EE/VS ratios ranging from 33% to  
335 40% exhibited similar tendencies in their VFA compositions, the main component of  
336 which was *n*-butyric acid (varying from 30% to 60%); after day 11, the propionic and  
337 valeric acid contents also increased. For samples with EE/VS ratios of 43–53%,  
338 earlier increases in propionic and valeric acid were observed on day 5, and the  
339 concentration of *n*-butyric acid was higher than that of valeric acid after days 25–30,  
340 while propionic acid was higher than acetic acid for samples with EE/VS ratios of  
341 33–40%. An accumulation of acetic, propionic and butyric acid was observed for the  
342 samples with lower EE/VS and high F/I ratios, resulting in lower biogas yields and  
343 pH values. An inhibition of propionic acid degradation was reported by (Raposo et al.,  
344 2006) when the acetic acid concentration was greater than 1400 mg/L. However, the  
345 present study revealed that biogas production was still inhibited at values lower than  
346 600 mg/L. Additionally, during the first stage, no major variations in the biogas yield  
347 were observed after day 3, during which time there was rapid VFA production;  
348 notably, the concentration of propionic acid did not exceed the threshold required for

349 methanogenic activity inhibition at 900 mg/L, as reported by (Wang et al., 2009). This  
350 finding suggested that the levels of VFAs required for inhibition may depend on  
351 feedstock compositions, which indicates that it is not feasible to define specific VFA  
352 inhibitory levels. Thus, anaerobic digester failure may be due to different operating  
353 parameters (e.g., characteristics of feedstock and inoculum).

354 However, considering the propionic acid to acetic acid ratio range, values greater  
355 than 1.4 indicated impending digester failure, which may serve as a satisfactory  
356 indicator for the beginning of organic overloading (Tang et al., 2008). As shown in  
357 Fig. 2 (F), the ratios increased to 1.0–1.2 on day 5, and higher values and longer  
358 durations were achieved for lower EE/VS and higher F/I ratios, implying low biogas  
359 production. The values varied from 0.60 to 0.79 at the end of the first stage and were  
360 accompanied by the start-up of biogas production. Thus, the low biogas yields in the  
361 first stage after day 5 (Fig. 1) may be due to inhibition caused by higher concentration  
362 of propionic acid, which is an undesirable intermediate product in the anaerobic  
363 process due to its slower metabolism via methanogenesis (i.e., the low conversion rate  
364 of propionic acid to acetic acid and H<sub>2</sub>/CO<sub>2</sub>) than acetate and butyrate (Zhang et al.,  
365 2005). In addition, neither methane content nor biogas yield showed a significant  
366 relationship with pH, but both exhibited a significant negative relationship ( $p < 0.01$ )  
367 with the VFA concentration. These findings disagree with a previous research report  
368 (Kawai et al., 2014) which found that the methane content demonstrated no  
369 significant relationship with the VFA concentration, but had a significant positive  
370 relationship with pH. This discrepancy may be due to differences in the substrate

371 characteristics, especially for the FW with higher EE content in this study. The VFA  
372 concentrations and compositions in the batch AD of FW may have been influenced by  
373 a synergistic effect of the EE/VS and F/I ratios.



374 **Fig. 2.** Effects of the EE/VS and F/I ratios on the VFA concentrations (A), VFA  
 375 compositions (B, C, D and E) and propionic acid to acetic acid ratios (F) with  
 376 increased retention time (RT).

377

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校对报告

380  
381 当前使用的样式是 [Energy Policy]  
382 当前文档包含的题录共61条  
383 有0条题录存在必填字段内容缺失的问题  
384 所有题录的数据正常

### 385 3.2.2. LCFAs

386 The major fatty acid constituents in the samples used in this study were unsaturated  
387 fatty acids, especially oleic acid (C18:1) and linoleic acid (C18:2), whereas palmitic  
388 acid (C16:0) was the main saturated fatty acid (Table 2). High concentrations of waste  
389 cooking oil in the initial substrate resulted in the high contents of these three  
390 constituents. According to the biogas yield pattern (Fig. 1), samples with low EE/VS  
391 and high F/I ratios had lower pH values, biogas yields and methane content, which  
392 may be explained by microbial injury due to the inhibition of LCFAs produced from  
393 the hydrolysis of lipids during the AD process, particularly in samples with a small  
394 amount of inoculum (e.g., F/I ratio higher than 0.80). In addition, the LCFA inhibition  
395 may impede the degradation of short-chain fatty acids during the hydrolysis and  
396 acidification processes (Hanaki et al., 1981; Miron et al., 2000; Palenzuela-Rollon,  
397 1999). The low biogas yield, low pH and high VFA concentration during the first  
398 stage of biogas production may also be attributed to the inhibition of palmitic acid  
399 (C16:0) produced via the  $\beta$ -oxidation of oleic acid (C18:1) and linoleic acid (C18:2).  
400 The inhibitory effects of unsaturated LCFAs are more toxic than those of saturated  
401 LCFAs (Lalman and Bagley, 2002). For example, C16:0 may create a physical barrier  
402 on microbial cells and hinder transfer processes, thus inhibiting biogas/methane  
403 production from propionate and butyrate (Pereira et al., 2005), especially in samples  
404 with lower waste cooking oil contents and higher F/I ratios.

405 However, without adjusting the pH values and alkalinity of the digestion system,  
406 the pH recovered concomitantly as the VFA concentration decreased, which was  
407 followed by an increase in biogas production in the second stage, indicating a  
408 reversible acidification process. No significant inhibitory effect was observed when  
409 the F/I ratios were less than 0.70, even when the EE/VS ratios increased to 50%. In  
410 addition, LCFA inhibition was reversible and could be eliminated after the depletion  
411 of biomass-associated LCFAs, namely, those with F/I ratios of 1.2, 1.0 and 0.8. These  
412 results also indicated that the digestion process was affected by synergistic effects  
413 between the inoculum ratios and the waste cooking oil content.

#### 414 3.2.3. Ammonia nitrogen

415 Digesters with higher F/I and lower EE/VS ratios exhibited higher final TAN  
416 concentrations ( $p < 0.01$ ), which ranged from 1345 to 1759 mg/L, as well as higher  
417 TAN concentrations corresponding to higher final VFA concentrations ( $p < 0.01$ ). The  
418 final FAN values ranged from 31.38 to 48.56 mg/L, and the highest FAN value was  
419 achieved for samples with EE/VS and F/I ratios of 36% and 1.00, respectively (Table  
420 6). The inhibitory thresholds of TAN and FAN have been reported to range from 1700  
421 to 2500 mg/L and from 400 to 1000 mg/L, respectively (Stams et al., 2003). The FAN  
422 levels were too low to inhibit the digestion process. However, it is important to note  
423 that the TAN concentrations in digesters with F/I ratios higher than 1.0 were near the  
424 reported inhibitory threshold. Additionally, an inhibition effect may appear for a FW  
425 sample with an EE/VS ratio higher than the set range and an F/I ratio within a certain  
426 range or for a FW sample with an F/I ratio higher than the set range and an EE/VS



427 ratio within a certain range. Hence, in this study, the slow biogas production rate  
428 during the first stage (Fig. 1) was mainly ascribed to the acidification caused by VFAs  
429 and LCFAs. However, lower EE/VS ( $p < 0.01$ ) and higher F/I ratios ( $p < 0.01$ ) may  
430 lead to higher final concentrations of VFAs and TAN. Thus, higher EE/VS ratios may  
431 not necessarily lead to inhibition via the accumulation of acids (VFAs and LCFAs) or  
432 alkalis (TAN, FAN), but lower F/I ratios may do ( $p < 0.01$ ).

### 433 *3.3. Digestate characteristics and relationships between process parameters*

434 The stability of the digestion process is important for maintaining sustainable  
435 anaerobic digester performance. Table 6 shows the characteristics of the final  
436 digestate including the pH, VFA concentration, VS reduction, and protein and EE  
437 reductions at the end of digestion, which indicates the stability of the AD system.

438 The final pH values (varying from 7.25 to 7.39) were all located in the preferred  
439 range for methanogenic activity. As one of the most important parameters for  
440 accurately controlling AD, the final VFA concentrations (0–0.53 mg/L) were very low  
441 for all of the samples at the end of the experiment, especially for samples with lower  
442 F/I ratios and higher EE/VS ratios ( $p < 0.01$ ), which is indicative of a complete  
443 digestion process. In the samples with lower EE/VS ratios and higher F/I ratios, the  
444 final distribution of the VFAs indicated higher concentrations of propionic and valeric  
445 acid and TAN and FAN, implying disturbances in the acetogenesis and  
446 methanogenesis pathways. Besides, only VFA ( $p < 0.01$ ) and TAN ( $p < 0.01$ ) were  
447 negatively correlated with EE/VS ratios ( $p < 0.01$ ).

448 The F/I ratios showed a significant negative correlation ( $p < 0.05$ ) with VS

449 reduction (25–43%), whereas EE/VS failed to show any significant effect. These  
450 findings indicated that the F/I ratio had an obvious influence on VS reduction. Lower  
451 F/I and higher EE/VS ratios were for a higher EE reduction (48 – 82%) compared  
452 with the opposite results for protein reduction (61–63%). Previous studies reported  
453 that higher lipid content contributed to the diffusion limitations imposed by layer  
454 surrounding the bacterial cells, thus increasing the lag-phase time, and lipid  
455 hydrolysis only occurred under methanogenic conditions (Miron et al., 2000) and  
456 higher lipid content contributed to higher VFA concentrations (Li et al., 2017).  
457 Besides, lipid hydrolysis only occurred under methanogenic conditions (Miron et al.,  
458 2000). Therefore, it could be concluded higher reduction of protein correlated with a  
459 higher lipid contents. Additionally, a high lag phase time ( $\lambda$ ) corresponded to  
460 increased protein reduction and less VS reduction compared to no significant effect on  
461 EE reduction, which was confirmed by significant positive correlations between the  $\lambda$   
462 values and the VFA ( $p < 0.01$ ) and TAN ( $p < 0.01$ ) concentrations. These findings  
463 suggest that a high VFA concentration during the reversible acidification process  
464 could be conducive to protein degradation.

465 High  $R_m$  values led to lower  $t_{90}$  values ( $p < 0.01$ ), lower final concentrations of  
466 VFAs and TAN ( $p < 0.05$ ) and shorter AD retention times ( $p < 0.05$ ) compared to  
467 higher EE reductions ( $p < 0.05$ ) and biogas yields ( $p < 0.05$ ). These findings suggest  
468 that the AD efficiency of FW is significantly influenced by the content of the waste  
469 cooking and the inoculum quantity. Therefore, to achieve the maximum recoverable  
470 biogas/methane yield from FW, it is necessary to provide organics with high

471 biomethane potential by increasing the EE/VS ratio and to conserve a sufficient  
472 amount of anaerobic microbes by starting the digestion assay at a very low F/I ratio.  
473 These findings are applicable for the dilution of waste cooking oil in feedstocks with  
474 higher inoculum amounts.

475 **Table 6.** Final characteristics of the digestates from FW with different F/I and EE/VS ratios.

EE/VS	33%	36%	40%	43%	46%	50%	53%
F/I	1.20	1.00	0.80	0.70	0.60	0.56	0.50
pH	7.28±0.02	7.37±0.01	7.28±0.02	7.39±0.16	7.26±0.01	7.25±0.10	7.28±0.01
VFA (mg/L)	0.53±0.01	0.34±0.01	0.15±0.01	0.38±0.00	0.31±0.00	0.30±0.03	0.00±0.00
Acetic acid (mg/L)	0.15±0.00	0.11±0.01	0.11±0.01	0.18±0.00	0.13±0.00	0.10±0.01	0.00±0.00
Propionic acid (mg/L)	0.29±0.01	0.14±0.00	0.05±0.00	0.01±0.01	0.00±0.00	0.01±0.01	0.00±0.00
Butyric acid (mg/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.10±0.00	0.00±0.00
Valeric acid (mg/L)	0.08±0.00	0.09±0.01	0.00±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.00±0.00
TAN (mg/L)	1759±26	1665±20	1567±25	1446±18	1501±17	1408±21	1345±49
FAN (mg/L)	41.94±6.75	48.56±4.82	37.35±5.90	44.12±6.89	34.21±9.18	31.38±7.12	32.07±4.89
VS reduction (%)	25	28	42	43	42	42	39
Protein reduction (%)	63	63	62	62	62	61	61
EE reduction (%)	48	56	61	60	67	82	86

### 476 3.4. Relationships between responses and independent variables

477 These findings suggested that both the substrate composition and the inoculation  
478 ratio co-affect the digestion parameters during AD. The EE/VS and F/I ratios were  
479 selected as independent variables, and nine corresponding parameters including  
480 organics reduction and performance stability parameters (VS, protein and EE  
481 reduction; methane content;  $t_{90}$ ; VFA and TAN final concentrations; and kinetic  
482 parameters) were selected as the dependent variables. The coefficients of the  
483 second-order polynomial models (Eq. (2)) corresponding to each dependent variable  
484 were evaluated and are listed in Table 7. The  $R^2$  values for these models ranged from  
485 0.839 to 0.999, indicating that the data can be well explained by these models, as the  
486  $R^2$  values are all greater than 0.75 (Naik and Setty, 2014). Additionally, in some cases,  
487 the terms  $(EE/VS) \times (F/I)$  and  $(EE/VS)^2$  were removed from the final polynomial to  
488 achieve lower  $p$  values (their coefficients were set to zero). All of the  $p$  values were  
489 lower than 0.05, so we can concluded that the model term was significant.

490 For FW digestion in practice, high biogas/methane production with a short  
491 retention time is always preferred as long as sustainable digestion is guaranteed.  
492 Considering the relationships between the performance and kinetic parameters and the  
493 F/I and EE/VS ratios, optimizing the F/I and EE/VS ratios to achieve high methane  
494 yield and biogas production efficiency without inhibition is necessary. The results of  
495 the second-order polynomials in terms of the F/I and EE/VS ratios in this study may  
496 also be used as a reference for experimental batch AD of FW to predict the system  
497 stability and to avoid inhibition (Table 7).

498 **Table 7.** Coefficients from the regression models.

Item	$M=M_0+aEE/VS+bF/I+c(EF/VS)^2+d(F/I)^2+e(EF/VS)\times(F/I)$						$R^2$	$p$
	$M_0$	$a$	$b$	$c$	$d$	$e$		
VS reduction	1.06	0.63	-0.67	-2.34	0.00	0.00	0.876	0.0257
Protein reduction	0.49	0.47	0.04	-0.53	0.00	0.00	0.839	0.0379
EE reduction	-4.82	7.22	4.57	0.00	-1.78	0.00	0.951	0.0065
Methane content	43.58	-131.23	-41.83	98.25	9.43	66.70	0.999	0.0012
$t_{90}$ (h)	11011.00	-36482.00	0.00	33364.00	0.00	0.00	0.981	0.0002
Final VFA (mg/L)	-36.07	118.11	8.15	-109.06	0.00	0.00	0.878	0.0251
Final TAN (mg/L)	-259.90	4928.20	806.20	-5005.60	0.00	0.00	0.912	0.0156
$R_m$ (mL/g VS h)	-101.50	286.20	39.70	-235.30	-9.70	0.00	0.988	0.0079
$\lambda$ (h)	-21656.00	24042.00	22815.00	0.00	-8819.00	0.00	0.873	0.0265

499 In addition, some problems associated with process instability can occur when the  
500 results from the BMP tests are applied in practice, as it is much easier to control their  
501 digestion parameters (e.g., feedstock composition, temperature, and anaerobic

502 environment) compared to pilot projects. A higher F/I ratio is always preferred due to  
503 cost and space savings, whereas a relatively larger EE/VS ratio is good for obtaining  
504 higher methane yield from a FW AD system. Therefore, to achieve the highest  
505 possible biogas/methane yield, a low F/I ratio (such as 0.5 – 0.7) combined with an  
506 ideal EE/VS ratio of slightly less than 43% is preferred.

#### 507 **4. Conclusions**

508 For EE/VS ratios lower than 40%, F/I ratios higher than 0.8 resulted in reversible  
509 acidification and possible LCFA inhibition along with lower biogas/methane yields  
510 and a longer lag phase. To minimize the possible inhibition caused by high EE/VS  
511 ratios during FW digestion, the F/I ratio should be lower than 0.70, which enabled the  
512 maintenance of a high biogas conversion ratio (82–94%) with high organics reduction  
513 and a short lag time (267–430 h). The optimum EE/VS and F/I ratios for the AD of  
514 FW are 43% and 0.70, respectively, as they resulted in the highest biogas yield and  
515 methane content and the largest VS reduction.

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520

521 E-supplementary data for this work can be found in e-version of this paper online  
522 ([Table A1](#)).

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