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2 **PILOT SCALE FERMENTATION COUPLED WITH ANAEROBIC**  
3 **DIGESTION OF FOOD WASTE - EFFECT OF DYNAMIC**  
4 **DIGESTATE RECIRCULATION**

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13

14 **Abstract**

15 The anaerobic digestion in double stage is a known and adopted system, but the process  
16 productivity and optimization still remain an aspect to investigate. The accumulation of organic  
17 acids (produced during fermentative metabolism) in the first stage generally decrease the pH below  
18 the optimal values (5.5). A pre-evaluation strategy by control charts for further pH control is  
19 proposed. The process combines in series the 1<sup>st</sup> Fermentation process and the 2<sup>nd</sup> Anaerobic  
20 Digestion process, using the recirculation of the anaerobic digestion effluent, rich in buffer agents,  
21 to control the pH in the 1<sup>st</sup> stage. The recycle ratio becomes a further operating parameter that  
22 should be properly managed. A proper management as dynamic recirculation flow allows to  
23 maintain the pH of the first phase to values higher than 5. Specific hydrogen production, specific  
24 methane production and volatile fatty acid production; 170 LH<sub>2</sub>/kgVS at 40% H<sub>2</sub>, 710 LCH<sub>4</sub> at 67%  
25 CH<sub>4</sub>, 14 gCOD/L VFA were obtained respectively.

26

27 **Keywords**

28 Hydrogen, methane, volatile fatty acids, process control, control chart, multivariate analysis

29

30

## 31 **1. INTRODUCTION**

32 Anaerobic digestion (AD) is a widespread and well known technology to treat organic waste of  
33 diverse stocks [1]. In the past it was considered as a system to manage the municipal waste.  
34 Nowadays the development of door-to-door separated waste collection makes the food waste an  
35 interesting source for energy and material production, and the AD becomes the main bio-refinery  
36 process able to answer to increasing energy demand. A further developing view of the AD process  
37 is to consider and manage it as a real production process [2]; therefore, the production should be  
38 maximized and its quality standardised.

39 AD involves different microorganism that through synergic way allow not only the production of  
40 methane but also other valuable products, hydrogen and volatile fatty acids [3]. In order to extract  
41 these different products, AD has to be split into two main phases [4][5][6].

42 The first phase of fermentation includes the step of hydrolysis, acidogenesis and part of the  
43 acetogenesis, instead the second phase substantially optimizes the last step, the methanogenesis.  
44 Therefore, through the optimization of the fermentation, hydrogen (gas), volatile fatty acid (liquid)  
45 and other low weight organic compounds such as alcohols and lactic acid [7] can be obtained. The  
46 VFAs can be used as external carbon source for biopolymers production, such as poly-hydroxyl-  
47 alkanoates [8][9].

48 Double stage AD is a known and adopted system, but the process productivity depends on HRT  
49 distribution between the two phases and pH control in the fermentation (1<sup>st</sup> stage). In fact, HRT and  
50 pH control can affect (inhibit or promote) several metabolic pathways and consequently the  
51 production of volatile fatty acids and hydrogen.

52 At the first two stages AD process have been suggested to adjust the physiological conditions  
53 requirements by the respective microbes involved in the different process stages. The optimal pH

54 values for the 1<sup>st</sup> and 2<sup>nd</sup> stage have, for example, been identified as pH 5.0-6.5 (for VFAs  
55 production), pH 5.5 (for Hydrogen production) and pH 7-8, respectively [10][11].

56 The accumulation of organic acids (produced during fermentative metabolism) in the first stage  
57 generally decrease the pH [12] below the optimal values. Recently some authors [13][14] proposed  
58 a strategy for pH control, coupling in series the 1<sup>st</sup> fermentation process with a 2<sup>nd</sup> anaerobic  
59 digestion process and using the recirculation of the anaerobic digestion effluent, rich in buffer  
60 agents, to control the pH in the 1<sup>st</sup> stage.

61 The recycle ratio becomes a further operating parameter that should be properly managed. The  
62 literature points out how to work with an excessive recirculation may result in a gathering of  
63 ammonia in the system and consequently into an inhibition of methanogenic [15] and  
64 hydrogenogenic processes [12]. Conversely recirculation ratios too low may be insufficient to  
65 control the pH of the reaction medium where the hydrogenogenic process occurs. Many process  
66 variables to control the process are involved, hence the process monitoring and fault detection are  
67 very important tasks in this biological engineering systems since they aim to ensure the success of  
68 the planned operations and to improve the productivity [2]. Since the complexity of AD process,  
69 many highly correlated variables are measured and should be subject to considerable misleading in  
70 a non-statistical data mining. Further, [16] stated that important information lies not only in any  
71 individual variable but also in how the variables change with respect to one another. On basis of  
72 these observations AD requires the application of analytical multivariate statistical methods.  
73 Multivariate analysis is a method to detect patterns and correlations in large datasets [17] such as  
74 the several parameters monitored in anaerobic process. This approach has been used for a long time  
75 in the chemical processing, but was only introduced into the industrial wastewater treatment plants  
76 in the late 1990s. However, our understanding of the multivariate statistical methods as evaluation  
77 to further control the AD processes is lacking in literature.

78 The aim of this work was the study of recirculation ratio effect by multivariate methods in order to  
79 further develop an optimized automatic control able to optimize the hydrogen and/or VFAs

80 production in the first phase, and methane generation in the second methanogenic one. Multivariate  
 81 analysis, focus on pH role, allowed to better understand the behaviour on recirculation ratio  
 82 variance.

83

## 84 2. MATERIAL AND METHODS

85 Initially, it has to be focused which region within the domain of possible values of the recirculation  
 86 ratio to consider. This way can eliminate a variable from the system. In the case to operate in the  
 87 region marked by high recirculation ratios, close to 1, the process control attention will be paid  
 88 exclusively to the content of ammonia in the system, that accumulates persistently. Conversely, in  
 89 the case to operate in the region characterized by low circulation ratio, next to 0.3, the goal will be  
 90 to verify if this ratio is largely sufficient to ensure an effective and lasting control of the pH in the  
 91 reaction medium of the fermentation process.

92 For this purpose, the experimental test was divided in three periods (RUNs): in RUN1 the  
 93 recirculation ratio was kept on 0.4 during overall period while in RUN2 and RUN3 it was kept  
 94 variable between 0.4 - 0.6 and 0.5 – 0.7 respectively, with a frequency of three weeks. In each trial  
 95 of this study we wanted to understand the influence of each recirculation ratio choice has exercised  
 96 alongside the fermentation process and the methanogenic process.

97 Table 1: Operational conditions applied during the experimental test

Parameters	units	RUN1	RUN2	RUN3
HRT 1 phase	d	3.3	3.3	3.3
HRT 2 phase	d	12.6	12.6	12.6
OLR 1 phase	KgTVS/(m <sup>3</sup> .d)	17	17	17
OLR 2 phase	KgTVS/(m <sup>3</sup> .d)	3.5	3.5	3.5
Recirculation Ratio	-	0.4	0.4 - 0.6	0.5 - 0.7

98

99           2.1.        *Analytical methods*

100   Substrates and digestates of both reactors were monitored three times a week in terms of total and  
101   volatile solids (VS), soluble (sCOD) and total chemical oxygen demand (COD), total nitrogen (TN)  
102   and total phosphorus (TP). Process stability parameters, namely pH, VFAs, free ammonia (NH<sub>3</sub>),  
103   total (T.ALK) and partial alkalinity (P.ALK) were checked daily. All the analyses, except for VFA  
104   and NH<sub>3</sub>, were carried out in accordance with the Standard Methods [18].

105   NH<sub>3</sub> was determined from the equilibrium relationship with N-NH<sub>4</sub><sup>+</sup> (AMM) in soluble in the  
106   aqueous fraction (Anthonisen et al. 1976). VFAs content was monitored using a gas chromatograph  
107   (GC) (Carlo Erba instruments) with hydrogen as gas carrier, equipped with a Fused Silica Capillary  
108   Column (Supelco Nukol TM, 15 m x 0.53 mm x 0.5µm film thickness) and with a flame ionization  
109   detector (200 °C). The temperature during the analysis started from 80 °C and reaches 200 °C  
110   through two other steps at 140 °C and 160 °C, with a rate of 10 °C/min. Samples were centrifuged  
111   and filtrated on a 0.45 µm membrane prior analysis. Biogas production was measured with two  
112   flowmeters (Ritter Company, drum-type wet-test volumetric gas meters), fitted on the reactors. The  
113   specific methane production (SMP) was determined using the methane concentration in biogas  
114   which was measured by a GC equipped with a HP-Molesieve column (30 m x 0.3 mm x 0.25 µm  
115   film thickness) employing thermal conductivity detection (TCD).

116

117           2.2.        *Data analysis*

118   According to [19] PCA is intended as a worthwhile chemometric technique when an effective  
119   reduction of the multidimensional space into few components is achieved, maintaining data  
120   variability. PCA provides an approximation of a dataset bringing back two matrices in reply: the  
121   matrix of scores and the matrix of loadings. In summary, these matrices capture the essential data  
122   patterns of the original dataset. Plotting the columns of the scores matrix gives a graph named score  
123   plot, where the relationship between observations is displayed and so clusters can be identified.  
124   Plotting the columns of the loading matrix returns another graph named loading plot, where the

125 relationship between variables is showed. In this way, the power importance analysis of variables to  
126 identify clusters is accomplished.

127

### 128 2.3. *Substrate and inoculum*

129 The anaerobic digested sludge used as inoculum for the methanogenic reactors (single stage and  
130 second phase) was collected in the WWTP located in Treviso (northern Italy) where a 2000 m<sup>3</sup>  
131 anaerobic digester treats the source collected biowaste at 35 °C. The sludge was acclimatized for  
132 one week to thermophilic temperature [20].

133 The substrate used in these experimental tests was the food waste from door-to-door collection of  
134 Treviso Municipality. The amount of total solids of biowaste used was 28% with a total volatile  
135 solids (TVS) on TS content of 92%. Regarding the content of nutrients, table 2 shows how food  
136 waste used in this study was characterized by an adequate nutrients ratio, particularly COD:N ratio  
137 with an average value of 41.

138 The fermentative reactor (first phase) was inoculated with food waste and water and then regularly  
139 fed with separately collected food waste and water in order to reach the volume required.

140

### 141 2.4. *Reactor set-up*

142 The reactors used were made of stainless steel AISI-304 with a working volume of 230 L for one-  
143 stage digester, and with reference to the two-stage process of 200 L for the fermentation unit and  
144 380 L for the digester unit. Mechanical anchor agitators ensured the mixing in order to maximize  
145 the degree of homogenization inside the reactor. The working temperature was set at 55°C ± 0.1  
146 (thermophilic temperature range) and maintained by an external jacket. The reactors were slightly  
147 pressurised at 0.01 atm.

148

### 149 2.5. *First stage (Hydrolysis) batch tests*

150 Batch tests were carried out to determine the hydrolysis step of food waste fermentation.

151 This part of the study was performed in order to investigate the hydrolysis in batch tests and the  
152 effective amount of volatile acids (VA) produced in relation to the pH. Hydrolysis potential batch  
153 tests (HPB) were carried out to determine the amount of VFAs and Lactic Acid (LA) production of  
154 the food waste with tap water in thermophilic condition.

155 First batch test was set up in triplicate mimicking the fermenter using different food waste to water  
156 ratio in order to determine the amount of VFAs and LA produced and observe the change in pH  
157 while the hydrolysis proceeds. Afterwards all the vials were flushed with a mixture of N<sub>2</sub> and CO<sub>2</sub>  
158 (80% and 20% respectively). These batch tests were run for one week. Everyday, samples were  
159 taken for pH, VFAs and LA analysis and hydrogen production. The pH was measured using pH  
160 meter and VFAs analysis performed. As suspect of lactic acid production, some representative  
161 samples were analysed with the HPLC. The procedure for lactic analysis, 2M H<sub>2</sub>SO<sub>4</sub> was used  
162 during sample preparation and the analysis was conducted using a HPLC (Ultimate 3000  
163 Dionex<sup>TM</sup>); HPLC on a Dionex Ultimate 3000-LC system (Dionex Corporation, Sunnyvale, CA,  
164 USA) with an Aminex<sup>®</sup> HPX-87H column coupled to a refractive index detector. As mobile phase  
165 H<sub>2</sub>SO<sub>4</sub> (4 mM) was used, with a flow rate of 0.6 ml/min at 60°C. All chromatograms were  
166 integrated using the Chromeleon software (Dionex Corporation).

167 The bio-hydrogen produced was also measured with the GC abovementioned. Total alkalinity was  
168 measured during the trial. Methane (CH<sub>4</sub>) production in the different vials was analysed by injecting  
169 gas samples from the headspace of each vial into the abovementioned GC for methane analysis and  
170 the batch vials were degasified whenever over-pressure of more than 1 bar was detected. Methane  
171 was analysed in order to understand when the hydrolysis in batch switched to a methanogenic  
172 activity.

173

### 174 **3. RESULT AND DISCUSSION**

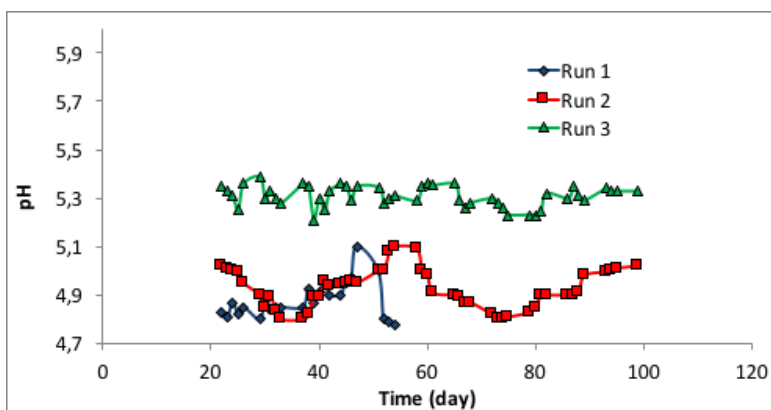
#### 175 *3.1. First phase*

176 The scope to find a suitable management of the process led to an accurate analysis the proper

177 recirculation ratio to adopt. To control the pH in the first stage by means of the digestate  
178 recirculation is advantageous economically, however it must be operated appropriately, otherwise  
179 the process itself leads to instability. [14] have shown how working with a high recirculation ratio  
180 would lead to an accumulation of ammonia in the system, able to inhibit both the methanogen  
181 consortium that the hydrogenogenic process. For this aim, three runs were tested; in each run a  
182 different strategy for the controlling of the pH were applied. In RUN1 the recirculation ratio was  
183 maintained to 0.4 for overall period, instead the RUN2 and RUN3 were operated with a variable  
184 recirculation ratio between 0.4 – 0.6 and 0.5 – 0.7 respectively, by varying this parameter  
185 alternately with a three-week frequency.

186 In figure 1, the trend of pH for three RUNs is presented.

187



188

189 Figure 1: first phase pH trend during the three RUNs.

190

191 The figure 1 shows how the RUN3 was the sole run where the pH was kept above 5 for the overall  
192 experimental trial. During the RUN1 the pH of the reaction medium has exceeded the value 5 only  
193 towards the end of the trial and moreover it was able to remain in this condition for a very short  
194 time. The low pH value of the reaction medium has adversely affected the hydrogenogenic activity  
195 reporting a low production of hydrogen (27 LH<sub>2</sub>/kgVS) and VFAs (7241 mg/L).

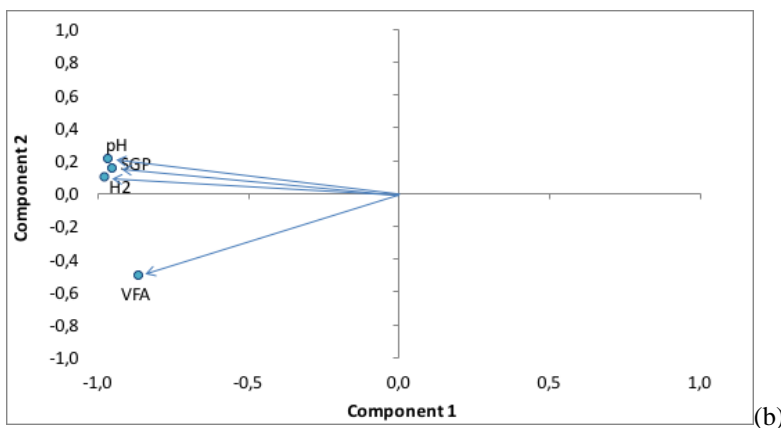
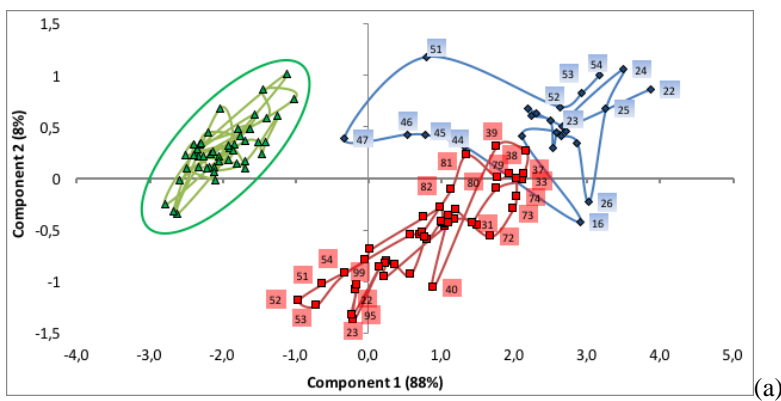
196 Observing the pH trend of RUN2 (figure 1) it is possible to note the average pH was lower than 5,  
197 values above 5 were detected only for a few days at the end of the 0.6 recycle period ratio. In other



198 words, during the RUN2 and RUN3 the alkalinity contribution provided by the digestate was not  
199 able to buffer acids produced during the fermentation phase. Also in this case the performance of  
200 the VFA and the hydrogen production were affected by the variability of the pH, 37 LH<sub>2</sub>/kgVS and  
201 9185 mg/L respectively.

202 To understand the different strategies effects of recirculation ratio applied on the production  
203 variables, principal component analysis (PCA) was used. PCA allows to reduce the  
204 multidimensional space into few components and therefore to study the relationship among  
205 variables and objects in the modelled space formed by principal components (PCs), saving data  
206 variability. Figure 2 shows the score and loading plots formed by the first and second PC (explained  
207 variances were designated in parenthesis).

208



211

211 Figure 2. Score (a) and loading (b) plot

212

213 Observing the loading plot (figure 2b) we can note how the pH was directly correlated with volatile

214 fatty acids and hydrogen production. These evidences are in according with [13] and [21]. The latter  
215 authors showed how the acetic acid can inhibit the metabolic activity of *Clostridium*  
216 *thermoaceticum* when the pH of reaction medium was lower than 5. Thus, the lower production of  
217 VFAs could be related to a detoxification mechanism of the cell to avoid the inhibitory effects.  
218 From the Score plot (figure 2a) we can just identify the cluster associated to the RUN3 (green  
219 ellipse) while a portion of the cases associated to the RUN1 and RUN2 showed themselves not  
220 distinguishable. Higher pH, VFAs yields, SGP and %H<sub>2</sub> characterize RUN3 than the other RUNs.  
221 Moreover, in RUN1 and RUN2 we note a higher and non-random variability than RUN3.  
222 For the study of the variability of the RUN1 and RUN2, the multivariate control chart approach was  
223 adopted [22].

224

225 RUN1 the control chart shows that in the period in which the reaction medium has exceeded value  
226 5, which is returned to the desired range, the process has highlighted very different characteristics  
227 compared to the previous condition. In particular, in the production of volatile fatty acids and the  
228 specific biogas production.

229 To understand the direction these variables have been taking in order to determine the shift of the  
230 process, a reduction of the dimensionality of the problem was performed, through the use of the  
231 principal components and the application of the Shewhart control chart.

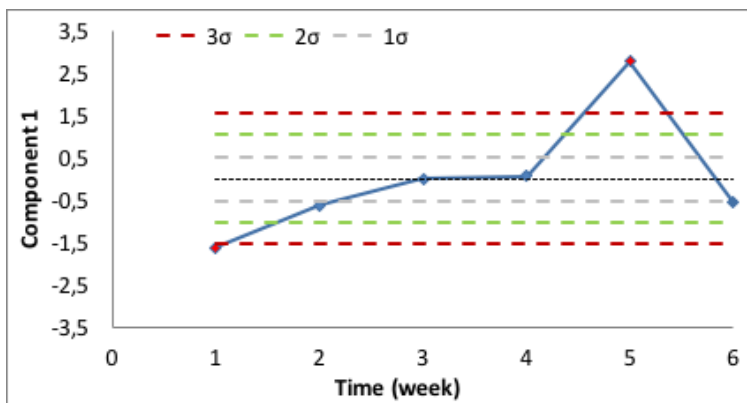
232 The first principal component extracted 77% of the total information and it was sufficient to  
233 describe the problem based on Rank Analysis criteria.

234 The x-bar chart RUN1 (x bar chart figure 3) confirmed an outside control signal which is much  
235 above the Control Limit ( $3\sigma$ ). Whereas the loadings of the first component we can underline as the  
236 out of control signal was due to high values of all the variables considered: pH 0.90 (first PC), VFA  
237 0.88, 0.83 SGP, H<sub>2</sub> 0.85.

238 On reaching the pH value of the first phase to values greater than 5, the fermentation process has  
239 highlighted an important change of condition. The system switched from a purely solvatogenic

240 condition, characterized by a low production of VFA and hydrogen, to an acidogenic one, vice  
 241 versa characterized by an increased production of volatile fatty acids and hydrogen. It is finally  
 242 noted that the increase of the hydrogen production is mostly due to the increase of the SGP, instead  
 243 of the hydrogen percentage in the biogas produced. In general, there was a positive correlation  
 244 between the pH, the specific hydrogen production and VFAs production, which confirmed what we  
 245 wanted to demonstrate.

246



247

248 Figure 3. X bar chart of the RUN1

249

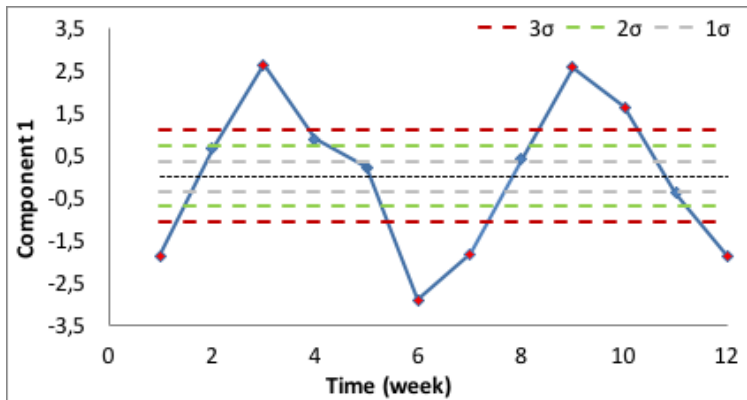
250 As a result of the accumulation of volatile fatty acids in the reaction medium, the alkalinity fed with  
 251 the recirculated digestate was not able to maintain a condition of pH to a value greater than 5. A  
 252 consequence of this the process switched back to the previous condition. In conclusion of this first  
 253 RUN1 it was not possible to maintain the pH of the fermentation process above 5 through the use of  
 254 a constant recirculation ratio equal to 0.4.

255 Through the RUN2 the first principal component describes the data to 90% and is therefore also in  
 256 this case sufficient to describe the process.

257 The x bar chart (figure 4) confirmed the hypothesis expressed in the previous RUN1. The  
 258 oscillatory trend of the principal component in the x-bar shows how the process does not respond to  
 259 a single distribution but two partially overlapping. On the basis of the considerations in the RUN1,  
 260 also in the RUN2 is possible to consider that the recirculation ratio strategy adopted in RUN2

261 swung the process in two different conditions, one acidogenic and one solvatogenic. Also in this  
262 trial is decisive the pH contribution to promote the two processes.

263



264

265 Figure 4. x bar chart of the RUN2

266

267 The fermentation process in RUN3 is most suitable for the production of VFA and hydrogen. The  
268 choice to operate with a variable recirculation ratio of 0.5 and 0.7 has allowed the accumulation of  
269  $\text{HCO}_3^-$  in the reaction medium. It has favoured the establishment of a buffer capacity which ensured  
270 the process stability even in the period of 0.5 recirculation ratio.

271 The trial RUN3 never left its optimum fermentation environment (cluster analysis figure 3), one  
272 that is within the pH above 5. Moreover, in this case a control chart does not show points out of  
273 control due to metabolic switch toward solvatogenic neither methanogenic conditions.  
274 Unlike other approaches, the pH of the first stage is maintained for the entire experiment above 5  
275 and it was not affected by the fluctuation of the recirculation ratio. Better performance on VFAs  
276 production (table 3 shows the main chemical - physical characteristics of the reaction medium, the  
277 stability parameters and production yields related to the fermentation process hydrogenogenic  
278 during RUN3), biogas composition and yields on the first stage.

279 The fermentation of RUN3 process is more suitable for the production of hydrogen and VFAs.

280

281 Table 3: stability parameters, chemical-physical characteristics and process yields for the I<sup>st</sup> phase RUN3

	Parameter	M.U.	Average $\pm$ St.Dev.	Min	Max
I° PHASE	TS	gTS/Kg	53 $\pm$ 5	46	61
	TVS	gTVS/Kg	44 $\pm$ 4	39	46
	COD	gO <sub>2</sub> /Kg	52 $\pm$ 9	41	63
	TKN	gN/Kg	1.6 $\pm$ 0.7	0.9	2.6
	P tot	gP/Kg	0.48 $\pm$ 0.10	0.45	0.50
	pH	-	5.3 $\pm$ 0.1	5.21	5.39
	VFA	mgO <sub>2</sub> /L	13,920 $\pm$ 488	11,616	14,957
	Total Ammonia	mgN-NH <sub>4</sub> <sup>+</sup> /L	687 $\pm$ 5	678	696
	SGP	Nm <sup>3</sup> /KgTVS	0.170 $\pm$ 0.010	0.165	0.172
	GPR	Nm <sup>3</sup> /(m <sup>3</sup> .d)	2.88 $\pm$ 0.04	2.72	2.95
	H <sub>2</sub>	%	40 $\pm$ 2	36	44
	CO <sub>2</sub>		52 $\pm$ 2	47	58
	CH <sub>4</sub>		7 $\pm$ 1	5	10

282

### 283 3.2. Hydrolysis batch tests

284 Different amounts of water were added to mimic a higher water saturation of the waste. The results  
 285 in table 4 reveal that the VFA concentration increased with adding less water, but the total amount  
 286 of VFA released from the waste decreased with lower water saturation. The highest VFA release  
 287 (1.52 g) corresponded to a conversion efficiency of the total organic matter (with a VS content of

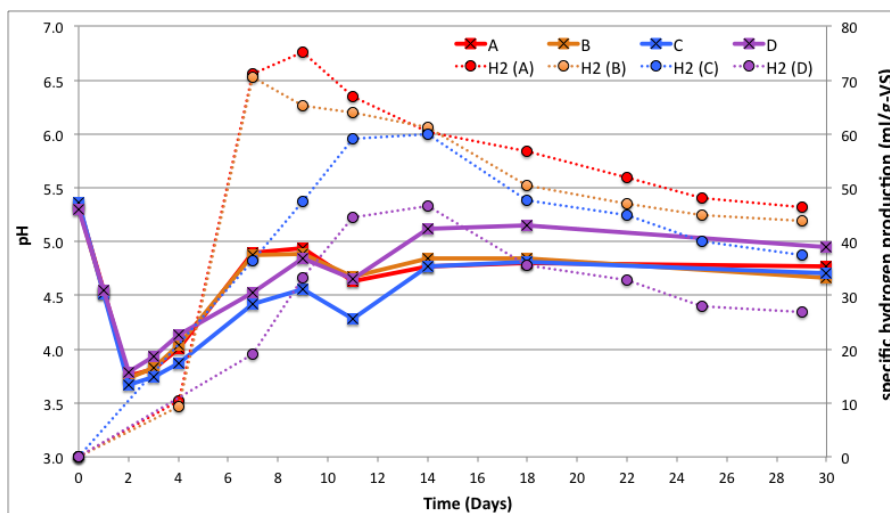
288 27%) into VFA of 24.8%.

289

290 Table 4. VFA release in batch hydrolysis setup with different amounts of water added

Set-up	OFMSW (g)	H <sub>2</sub> O (mL)	Waste/percolate ratio	VFAs (g/L)	VFA (g)
A	23.4	300.0	7.8%	5.05	1.52
B	23.4	221.0	10.6%	6.17	1.36
C	23.4	158.0	14.8%	7.55	1.19
D	23.4	78.8	29.7%	11.30	0.89

291



292

293 Figure 5. Hydrogen production during the second HBT trials.

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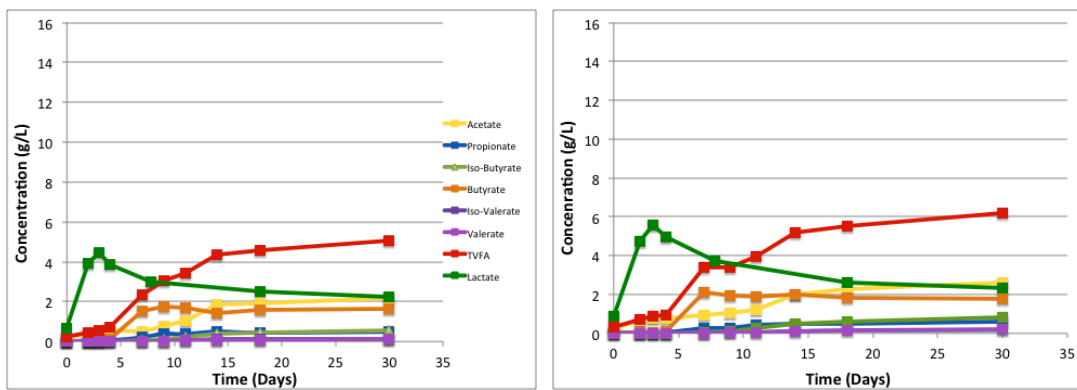
295 The production of hydrogen was detected and the data showed great variability amongst the batch  
296 set-up. All samples showed an increase in hydrogen production over the first 10 days and eventually  
297 a decrease. No hydrogen production was detected when pH was below 4.5.

298 For all the samples, the pH fell rapidly in the first 2-3 days to around 3.70 before it rose again and  
299 reached a plateau (figure 5). The anomalous pH drops for all samples around day 10 could be due to  
300 pH calibration error. The overall pH of each setting, after 30 days, has no significant differences;  
301 data after 30 days showed a standard deviation of 0.13. As expected, there is evidence of high

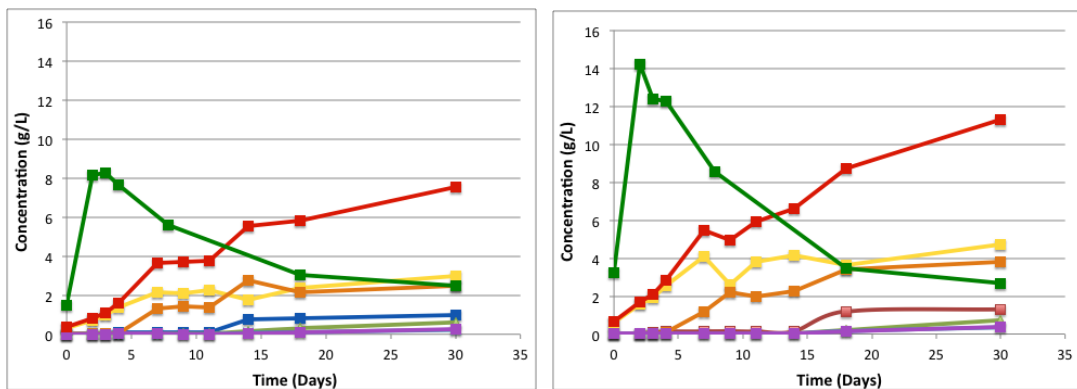
302 production of lactic acid in all samples of the first 4 days that is responsible for the rapid drop in pH  
 303 of all batch setup (figure 5). The maximum concentration of lactic acid ranges from 4.5 to 14 g/L.  
 304 The more concentrated the food waste biomass is, the higher the concentration of lactic acid. There  
 305 is a high possibility that there would be lactic acid production taking place in the reactor setup.  
 306 It is therefore important to not have too high the organic loading in the reactor setup as this would  
 307 result in unwanted lactic acid production or to control the pH of the fermentation phase above pH 5.  
 308 There is a high possibility that lactic acid is produced in the reactor setup below pH 5 [21].  
 309 However, it can be seen that the high lactic acid concentration for every batch setup correlate to a  
 310 pH below 4.5 [23]. Therefore, lactic acid production could have already been avoided in the reactor  
 311 as the pH will be keep strictly in the range of 5-5.5, by a dynamic digestate recirculation.

312

313



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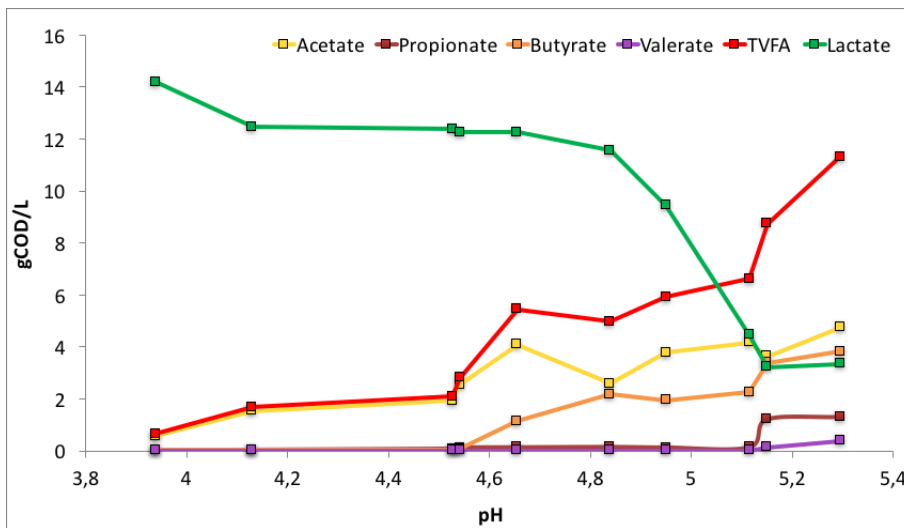
315 Figure 6. Lactic acid and VFAs production of the four hydrolysis batch tests.

316

317 Likewise, the VFA concentration increased as the water concentration decreased. The concentration  
 318 of VFA for A, B, C, D after 30 days were 5.05 g/L, 6.17 g/L, 7.55 g/L and 11.30 g/L respectively.

319 However, the VFAs in grams for A, B, C and D were 1.51 g/L, 1.36 g/L, 1.19 g/L and 0.89 g/L.

320



321

322 Figure 7. Lactic Acid and Volatile Fatty Acid production related to pH tendency on HBT.

323

324 In figure 7, the volatile acids on pH function are reported. It is possible to underline in what way  
325 below pH 5 the lactic acid is predominant, on the contrary the VFAs production, particularly acetic  
326 acid and butyric acid is noticeably incremented at pH values higher than 5.  
327 It is demonstrated how above pH 5 volatile fatty acid production is enhanced, this is well correlated  
328 with literature data of [24].

329

### 330 3.3. Second phase

331 Figure X shows the results of analysing agglomerative hierarchical cluster.

332 The objects of the dendrogram from 1-24 match RUN 1, 25-73 RUN 2, 74-120 RUN 3.

333 The algorithm performed the fusion of objects considered by increasingly larger cluster size as the  
334 distance among the objects (decreasing similarity).

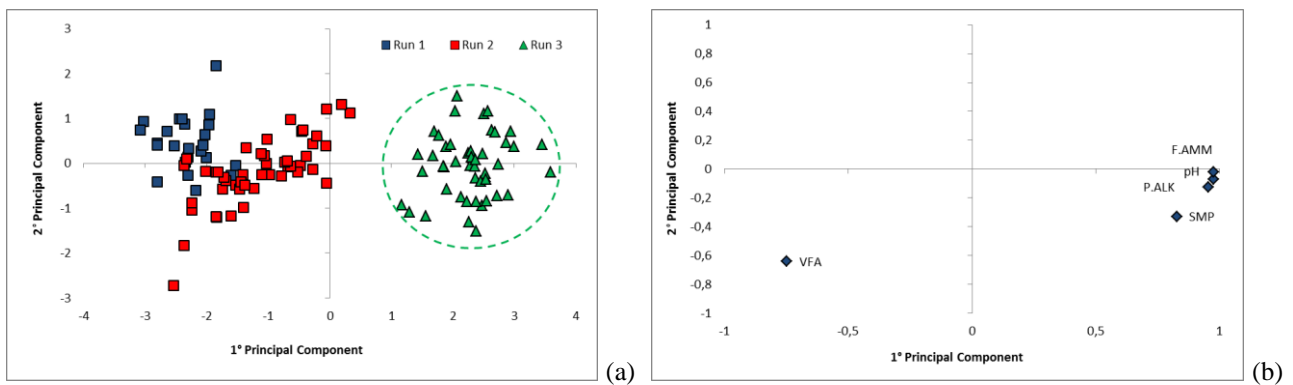
335 One grouping on the RUN 3 is detectable. The other two groups (RUN 1, 2) are indistinguishable.

336 This analytical methodology highlights how recirculation ratio 0.4 and ratio 0.4 – 0.6 (variable) did  
337 not produced a visible change in the characterization of the methanogenic process. On the other



338 hand, the recirculation ratio of RUN 3 allowed to obtain a distinguishable process among the  
339 previous RUNs, based on the 5 variables considered (pH, NH<sub>3</sub>, alkalinity, SMP, VFA). The  
340 obtained result underlined that it was necessary to analyse the role of these variables that helped to  
341 distinguish the methanogenic process RUN 3. This agglomerative hierarchical analysis does not  
342 allow to obtain this information, which is possible to obtain through the principal component  
343 analysis instead. By principal components analysis it is in fact possible to comprehend the relevance  
344 of the original variables have had in the clusters analysis by Loading Plot graph.

345



346

347 Figure 9. Score plot (a) and Loading plot (b) of the second methanogenic phase RUNs.

348

349 The Score Plot allows the first principal component, which extracts 78% of the information overall,  
350 to show a clear separation among the observations on the RUN3 among the other RUNs.

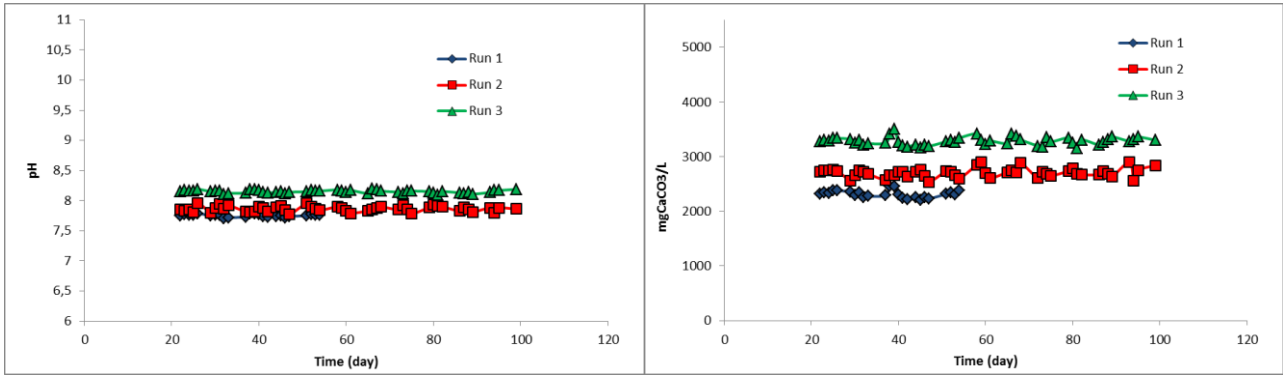
351 The analysis of the Score plot jointly the Loading Plot highlights the role of the five variables.

352 As previously, also by means of the use of the main components it was able to isolate only the  
353 cluster relative to the RUN3. Interpreting jointly the Score plot with the plot Loading is possible to  
354 notice how the second stage of RUN3 is distinct from the remaining tests; it is characterized by  
355 higher pH, partial alkalinity and higher SMP and minor VFAs content, which indicates a better  
356 efficiency of the process.

357 As regards the second phase, in the next figure the stability parameters trends are reported.

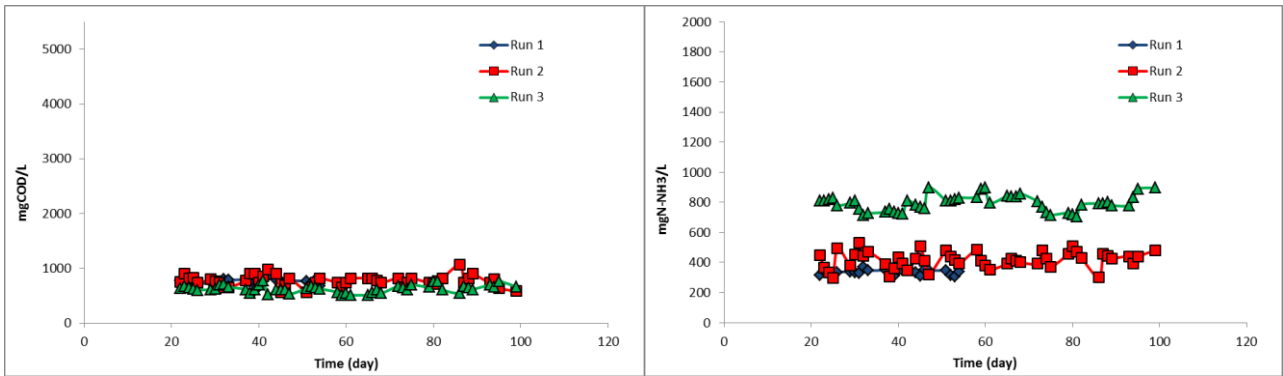
358

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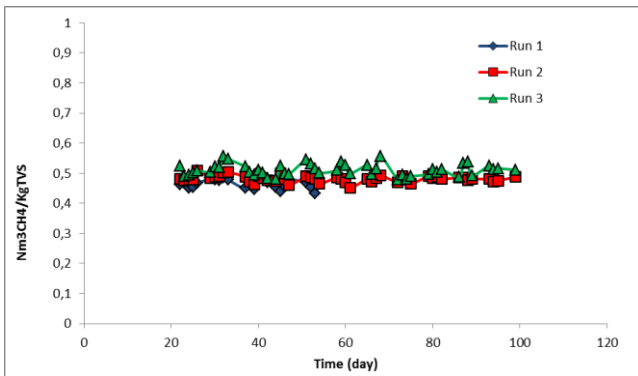
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364 Figure 8. Stability parameters during the three RUNs in the second methanogen phase.

365

366 Table 5 shows the main chemical - physical characteristics of the reaction medium, the stability  
367 parameters and the production yields related to the methanogenic process during RUN3.

368

369 Table 5. Stability parameters, chemical-physical characteristics and process yields for the II<sup>nd</sup> phase RUN3

Parameter	M.U.	Average ±	Min	Max
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			St.Dev.		
II° PHASE	TS	gTS/Kg	23.2 ± 4	26	30
	TVS	gTVS/Kg	16 ± 3	10	21
	COD	gO <sub>2</sub> /Kg	20 ± 2	19	23
	TKN	gN/Kg	1.5 ± 0.2	1.0	1.8
	P tot	gP/Kg	0.21 ± 0.01	0.10	0.25
	pH	-	8.15 ± 0.10	8.10	8.20
	P. Alkalinity	mgCaCO <sub>3</sub> /L	3,283 ± 73	3,145	3,498
	T. Alkalinity		5,256 ± 50	5,157	5,376
	VFA	mgO <sub>2</sub> /L	631 ± 72	449	781
	Total Ammonia	mgN-NH <sub>4</sub> <sup>+</sup> /L	1,539 ± 148	1,290	1,885
	Free Ammonia	mgN-NH <sub>3</sub> /L	794 ± 52	706	898
	SGP	Nm <sup>3</sup> /KgTVS	0.75 ± 0.02	0.71	0.79
	GPR	Nm <sup>3</sup> /(m <sup>3</sup> .d)	2.50 ± 0.10	2.37	2.77
	CH <sub>4</sub>	%	67 ± 2	64	70
	CO <sub>2</sub>		32 ± 2	29	35

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#### 4. CONCLUSIONS

373

In conclusion, Cluster analysis allowed to understand how among the three processes studied only

374 the RUN3 has shown a different condition, in the direction of a better efficiency of the process, both  
375 from yields point of view and through stability process parameters, in particular the higher  
376 alkalinity amount in the reaction medium.

377 A proper management of the recirculation allows to maintain the pH of the first phase to values  
378 higher than 5. It allows to foster metabolic hydrogenogenic processes and it seems also to improve  
379 the environmental conditions occurring the methanogenic processes, in particular by increasing the  
380 alkalinity of the reaction medium.

381

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385

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