



**HAL**  
open science

## Robust operation through effluent recycling for hydrogen production from the organic fraction of municipal solid waste

Florian Paillet, Carole Barrau, Renaud Escudié, Nicolas Bernet, Eric Trably

### ► To cite this version:

Florian Paillet, Carole Barrau, Renaud Escudié, Nicolas Bernet, Eric Trably. Robust operation through effluent recycling for hydrogen production from the organic fraction of municipal solid waste. *Bioresource Technology*, 2021, 319, pp.124196. 10.1016/j.biortech.2020.124196 . hal-03102599

**HAL Id: hal-03102599**

**<https://hal.inrae.fr/hal-03102599>**

Submitted on 17 Oct 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Robust operation through effluent recycling for hydrogen production from the**  
2 **organic fraction of municipal solid waste**

3 Florian Paillet<sup>a,b</sup>, Carole Barrau<sup>a</sup>, Renaud Escudié<sup>b</sup>, Nicolas Bernet<sup>b</sup>, Eric Trably<sup>b\*</sup>

4 <sup>a</sup> TRIFYL, Route de Sieurac, 81300 Labessiere-Candeil, France

5 <sup>b</sup> INRAE, Univ Montpellier, LBE, 102 avenue des Etangs, 11100, Narbonne, France

6 \*Corresponding author: [eric.trably@inrae.fr](mailto:eric.trably@inrae.fr)

7 **Abstract**

8 The stability of fermentative hydrogen production from the organic fraction of municipal  
9 solid waste (OFMSW) was evaluated in this work using a strategy of effluent recycling.  
10 Three pretreatment conditions were applied on the recycled effluent: a) no heat shock  
11 treatment, b) one initial heat shock treatment (90°C, 30 min) and c) systematic heat shock  
12 treatment at the beginning of each fermentation. When a systematic heat shock was applied,  
13 a maximal hydrogen yield of 17.2±3.8 mLH<sub>2</sub>/gVS was attained. The hydrogen productivity  
14 was improved by 331% reaching a stable value of 1.51±0.29 mLH<sub>2</sub>/gVS/h, after 8 cycles of  
15 effluent recycling. This strategy caused a sharp decrease of diversity with stable co-  
16 dominance of hydrogen- and lactate-producing bacteria, ie. *Clostridiales* and  
17 *Lactobacillales*, respectively. For the other conditions, a sharp decrease of the hydrogen  
18 yields was observed showing the importance of applying a heat shock treatment for optimal  
19 hydrogen production with effluent recycling.

20

21 **Keywords** : Biohydrogen, Dark fermentation, Effluent recycling, Organic Fraction of Municipal  
22 Solid Waste, Stability

23

## 24 1. Introduction

25 The Organic Fraction of Municipal Solid Waste (OFMSW) is a highly available resource  
26 that can be valorized through biological processes such as dark fermentation. Over the past  
27 decade, hydrogen production by dark fermentation has been investigated as an interesting  
28 way of valorization, as H<sub>2</sub> is considered as an important carrier for the next-generation  
29 technologies (Dawood et al., 2020). However, one of the main limiting factors of  
30 fermentative biohydrogen production is the low yield of organic matter conversion that can  
31 make the process not economically reliable. To improve the viability of such process, the  
32 most interesting solution is to couple dark fermentation with anaerobic digestion,  
33 generating a mixture of H<sub>2</sub>/CH<sub>4</sub> (5-20% of H<sub>2</sub>) so-called Biohythane (Meena et al., 2020).  
34 Such coupling consists of a separation of acetogenesis/acidogenesis and methanogenesis  
35 making possible an optimization of each step for improving the total energy recovery. In  
36 addition, the hydrogen production in the first step does not have a significant negative  
37 impact on the methane yield compared to a single stage process, making hydrogen a pure  
38 gain of energy in the two-stage process (Hans and Kumar, 2019). Nonetheless, one  
39 limitation in coupling these two processes is the low total solid (TS) content to be applied  
40 in dark fermentation prior to the anaerobic digestion (*i.e.* methane production) step. As  
41 already reported, high hydrogen yields were observed for TS lower than 15% (Ghimire et  
42 al., 2018). In contrast, anaerobic digesters using OFMSW as substrate are usually operated  
43 at high TS content (>15%), in particular when solid-state anaerobic digestion technologies  
44 are implemented. In this context, the liquid part (so called “effluent”) from dark  
45 fermentation could be removed to increase the TS of the substrate used for the anaerobic  
46 digestion. The collected effluent can here be recycled to the next fermentation step, which

47 is an interesting and innovative solution to supply in water the feeding of the first process.  
48 Indeed, OFMSW are characterized by a low water content, with a TS of about  $27.2\pm 5.8\%$   
49 (Campuzano and González-Martínez, 2016). In addition, effluent recycling can ensure the  
50 inoculation of the dark fermenter with a well-adapted microbial community. Several studies  
51 have already highlighted the positive impact of the use of effluent recirculation from a  
52 methanogenic reactor to dark fermenter inoculation (O-Thong et al., 2016). In particular,  
53 Zhang et al. (2007) showed the benefit of recirculating the methanogenic effluent on an  
54 hydrolysis-acidogenic step by improving the total extracellular enzyme activities. This  
55 strategy could be particularly adapted to dark fermentation by recycling the effluent after a  
56 cycle of hydrogen production in discontinuous systems. However, effluent recirculation  
57 may also promote the accumulation of soluble chemicals such as ammonia nitrogen, VFAs  
58 and total ions that can hinder the hydrogen producing bacteria (Ariunbaatar et al., 2015;  
59 Cavinato et al., 2012; Paillet et al., 2019). To avoid hydrogen consumption or the  
60 development of non-H<sub>2</sub>-producers bacteria, several physico-chemical pretreatments have  
61 already been proposed such as a pH shock (Jang et al., 2015), a chemical treatment (Zhu  
62 and Béland, 2006) or a heat shock treatment, the latter being the simplest, the less  
63 expensive and the most effective method (Ghimire et al., 2015).

64 The aim of this study was to investigate different pretreatment conditions for recycling the  
65 effluent of dark fermentation in successive batch reactors, in order to adapt the microbial  
66 communities while avoiding inhibition of hydrogen production. Different modes of heat-  
67 shock treatments were applied on the liquid phase (one initial heat shock and systematic  
68 heat shock before each fermentation) to reduce the development of methanogens.

69 Accumulation of potential inhibitors was assessed to evaluate the long-term robustness of  
70 the dark fermentation process.

## 71 **2. Materials and Methods**

### 72 **2.1. Feedstock and inoculum preparation**

73 Organic Fraction of Municipal Solid Waste (OFMSW) was freshly prepared according to  
74 the average composition of OFMSW collected in France on a yearly basis (MODECOM™,  
75 1993). The proportions of each component are described in Table 1. Meat, rice, potatoes  
76 and coffee grounds were cooked and mixed with yogurt and bread. Garden waste, paper  
77 and cardboards were shredded and sieved at 1 cm. Total solid (TS) and volatile solid (VS)  
78 contents of the freshly prepared OFMSW were  $0.74\pm 0.01$  gTS/g and  $0.63\pm 0.01$  gVS/g,  
79 respectively. The anaerobic inoculum corresponded to a sample of anaerobic lagoon  
80 treating leachates of methanogenic storage cells from a MSW landfill. The pH was 7.6, the  
81 conductivity  $23.9\pm 2.6$  mS/cm, and the TS and VS contents were  $0.0205\pm 0.0003$ gTS/g and  
82  $0.0070\pm 0.0002$  gVS/g, respectively ( $\text{NH}_4^+$ :  $2.4\pm 0.2$  g/L). Heat-shock treatment, was  
83 performed in a stirred bottle at 90°C for 30 min in order to inactivate methanogens  
84 (Ghimire et al., 2015).

### 85 **2.2. Experimental set-up**

86 All the experiments were carried out in a 6 L reactor made of polyvinyl chloride (PVC)  
87 manufactured for the study. Each batch reactor was performed at 37°C (Fisher scientific,  
88 Polystat 24, USA) and filled with 3 L of inoculum and 663.6 g (*i.e.*, 420.7 gVS) of freshly  
89 prepared OFMSW to obtain a total solid (TS) content of 15 %. Operating the fermenter at  
90 high solid contents increases productivity (mLH<sub>2</sub>/Lreactor/day), and reduces the amount of  
91 leachate addition. In accordance with preliminary tests, a TS content of 15% was selected

92 as it allows to obtain similar hydrogen yields at lower MS contents. pH was not regulated,  
93 and the experiments were stopped when hydrogen accumulation stopped (3-4 days). The  
94 first reactor of one initial heat shock experiment was operated for a longer time (5.3 days)  
95 because hydrogen production was still detected at day 5. At the end of experiments, the  
96 liquid fraction of the fermentate, called effluent, was sieved at 1mm and separated from the  
97 solid fermentate. The effluent was then used for a new fermentation batch. Additional  
98 leachate, stored in cold chamber (4°C), was added to the recycled effluent and the  
99 OFMSW, to adjust the total volume at 3 L with a TS content of 15%.

### 100 **2.3. Process operation**

101 A schematic representation of the three experimental modes is provided in Fig. 1. A control  
102 was carried out without any heat shock treatment during the experiments. Two different  
103 modes of heat shock treatments were performed on the liquid phase. The condition named  
104 “one initial heat shock” consisted of one unique heat treatment on the leachate used for the  
105 first fermentation. The recycled effluent and the additional leachate for the next  
106 fermentation were then used without any heat treatment. The condition named “systematic  
107 heat shock” consisted to a systematic heat treatment of the liquid part of the dark fermenter.  
108 This liquid part was composed of only leachate for the first fermentation cycle, and a  
109 mixture of recycled effluent and additional leachate for the next cycles (Fig. 1). The ratio  
110 corresponding to “additional leachate/effluent” was  $51 \pm 8.3\%$  whatever the treatment  
111 conditions, due to the similar feedstock applied in all experiments.  
112 The control and the experiment with initial heat shock treatment were carried out on 6  
113 successive batch reactors (6 cycles, 5 effluent recycling). The systematic heat shock  
114 experiment was performed during 16 successive batch reactors (16 cycles, 15 effluent

115 recycling). For the three modes investigated, each reactor was named chronologically, eg.  
116 the first reactor was called reactor n°1, second reactor n°2 etc. After each feeding operation,  
117 the reactor headspace was purged with pure nitrogen gas to remove oxygen traces. 2 mL of  
118 liquid phase was sampled and stored at -20°C at the beginning and at the end of each batch  
119 for further analyses of metabolites and microbial composition. The pH, conductivity and  
120 ammonia nitrogen concentration were measured at the beginning and at the end of each  
121 batch.

## 122 **2.4. Analytical methods**

123 The gas composition was periodically measured (every 6 h) with an automated multiplexed  
124 micro-gas chromatograph ( $\mu$ GC R3000, SRA instrument, France) equipped with two  
125 analytical capillary columns to monitor on-line the gas production. The first column was  
126 dedicated to carbon dioxide analysis (10 m length and 0.32 mm diameter) with argon as  
127 carrier gas at a pressure of 30 PSI. The second column was dedicated to oxygen, hydrogen,  
128 nitrogen and methane analysis (8 m length and 0.32 mm diameter) with helium as carrier  
129 gas (20 PSI). The injector and column temperatures were 90°C and 80°C, respectively  
130 (Motte et al., 2013). The total volume of gas produced was measured by a gas meter  
131 (MilliGascounter® MGC-1, Ritter, Germany).

132 Metabolites were measured at the beginning (on the mixture of recycled effluent and  
133 additional leachate) and at the end of each batch test, by using a high-performance liquid  
134 chromatographic chain composed of an automatic sampler (Waters, 717 plus Autosampler),  
135 a pre-column (Micro guard cation H refill cartridges, Bio-rad) and a Aminex HPX-87  
136 column set at 35°C. The carrier eluent corresponded to a 4mM sulfuric acid solution at a  
137 flow rate of 0.4 mL.min<sup>-1</sup> (Motte et al., 2013).

## 2.5. Microbial community analysis

For microbial analyses, samples were collected at the end of each dark fermentation. DNA was extracted and purified with the Fast DNA SPIN kit for soil in accordance with manufacturer's instructions (MP Biomedicals). DNA quantity and purity were assessed by spectrophotometry (Infinite NanoQuant M200, Tecan). The V4 and V5 regions of the 16S rRNA genes were then amplified and sequenced by Illumina MiSeq (get.genotoul.fr) and sequences were analyzed.

## 2.6. Data analysis and calculation

Hydrogen production performances (i.e., hydrogen yield, maximal hydrogen production rate and lag phase) were calculated by fitting the cumulative hydrogen production curves with a modified Gompertz based-model (Eq. 1):

$$H(t) = H_{max} \times \exp \left\{ -\exp \left[ \frac{R_{max} \times e}{H_{max}} (\lambda - t) + 1 \right] \right\} \quad (\text{Eq. 1})$$

where  $H(t)$  is cumulative hydrogen volume (mL) at time  $t$  (h),  $H_{max}$  is the maximum hydrogen potential (mL),  $R_{max}$  is maximum hydrogen production rate (mL/h),  $\lambda$  is the lag-phase and  $e$  is the  $\exp(1) = 2.718$ . By considering the initial amount of VS in the substrate, it is possible to estimate the maximum hydrogen yield (mL $H_2$ /gVS) and the maximum hydrogen productivity (mL  $H_2$ /gVS/h). The values of  $H_{max}$ ,  $R_{max}$ , and  $\lambda$  were estimated using grofit R package (v 1.1.1-1).

All statistical analyses were performed with R software (v3.6.2) using RStudio (v1.2.5033). The "FactoMiner" (v2.1) package was used for PCA analysis, and the package "factoextra" (v1.0.6) for graphic visualization.

## 3. Results and discussion



### 3.1. Hydrogen production

160  
161 Three parameters representative of hydrogen production performances, i.e. maximum  
162 hydrogen yield (mLH<sub>2</sub>/gVS), hydrogen lag phase (h) and maximum hydrogen productivity  
163 (mLH<sub>2</sub>/gVS/h) were estimated for each batch test. Fig. 2 presents these parameters over the  
164 total duration of the experiments for the three different modes of recycled effluent  
165 treatment: i) no heat shock, ii) one initial heat shock and iii) a systematic heat shock  
166 treatment. For the mode with no treatment (control), the highest H<sub>2</sub> yield was observed  
167 during the first batch cycle at 18.8 mLH<sub>2</sub>/gVS and was close to the value reported by  
168 Alibardi and Cossu. (2015) using OFMSW (~30±20 mLH<sub>2</sub>/gVS). The production of  
169 hydrogen varied significantly in the second and third reactors (H<sub>2</sub> yield of 10.8 and 15.9  
170 mLH<sub>2</sub>/gVS, respectively). The last three reactors (N°4, 5 and 6) showed a continuous  
171 decrease of the H<sub>2</sub> yield. In the last reactor (*i.e.* after 5 cycles of effluent recycling), almost  
172 no H<sub>2</sub> production was detected (0.4 mLH<sub>2</sub>/gVS). Regarding the hydrogen lag phase, the  
173 value measured in the first reactor was 22.4 h and decreased after the first effluent recycling  
174 cycle reaching a constant value for the next reactors, at 7.5±0.5 h, which corresponded to a  
175 reduction of 66.5%. In terms of hydrogen productivity, an unstable value was found  
176 ranging from 0.57 to 1.23 mLH<sub>2</sub>/gVS/h during the first four reactors, before a drastic drop  
177 for the last two batches at 0.20 and 0.03 mLH<sub>2</sub>/gVS/h, respectively. For the mode  
178 consisting of one initial heat shock treatment, a trend similar to the control was observed in  
179 terms of H<sub>2</sub> yield. The best H<sub>2</sub> yield was observed in the first reactor with 10.5 mLH<sub>2</sub>/gVS.  
180 This value is slightly lower than in the control and can be explained by the inoculum  
181 pretreatment that could negatively affect the production of hydrogen by generating stressful  
182 conditions for the microbiota as reported by Luo et al., (2010). Then a continuous decrease

183 was observed until a low production for the last reactor (2.6 mLH<sub>2</sub>/gVS). The hydrogen lag  
184 phase in the first reactor was 25.3 h and decreased after the first effluent recycling, reaching  
185 an average value of 7.9±2.0 h (reduction of 68.8%). When a systematic treatment was  
186 performed on the liquid phase (*i.e.* on the leachate used for the first reactor and then on the  
187 mixture of recycled effluent and additional leachate), a constant hydrogen production with  
188 an averaged value of 17.2±3.8 mLH<sub>2</sub>/gVS was observed during 16 successive batch  
189 reactors. The average hydrogen yield measured was similar to the performances found in  
190 the literature for OFMSW when using heat shock treatment of the inoculum. In the study of  
191 Favaro et al. (2013), a production of hydrogen of 23.4±2.9 mLH<sub>2</sub>/gVS was reported when  
192 heat shock treatment at 100°C for 4 h was applied using OFMSW (160±10 gTS/L). The lag  
193 phase was also very stable around 8.8±2.6 h. Interestingly, the maximum hydrogen  
194 productivity also increased from 0.35 mLH<sub>2</sub>/gVS/h from the first reactor to a stable value  
195 of 1.51±0.29 mLH<sub>2</sub>/gVS/h after 8 cycles of effluent recycling. Comparing the different  
196 treatment modes, no heat shock and one initial heat shock treatments showed a similar  
197 behavior with a global decrease of hydrogen production until almost no production after 6  
198 successive batch cycles (H<sub>2</sub> yield of 0.4 and 2.6 mLH<sub>2</sub>/gVS, respectively). With these two  
199 modes, methane was rapidly detected. Table 2 presents the time to reach the maximum  
200 hydrogen productivity and the time where significant methane accumulation was first  
201 detected. In the control, methane was detected after 51 h during the first batch. Thereafter,  
202 for reactors 4, 5 and 6, methane was produced at the same time than the maximum  
203 hydrogen productivity (17.7, 12.0 and 8.1 h, respectively). The decrease of hydrogen  
204 production can therefore be correlated with hydrogen consumption through methanogenesis  
205 (Ghimire et al., 2015). Four moles of hydrogen can theoretically be converted into one

206 mole of methane by hydrogenotrophic methanogens (Xu et al., 2019). For the mode  
207 consisting in one initial heat shock, methane was also detected in the first batch but with a  
208 longer delay (127.9 h) showing the benefit of heat shock treatment of the inoculum.  
209 However, after the first effluent recycling, methane was produced faster (23.32 h) and  
210 closer to the time of maximum hydrogen production. Thus, a heat shock treatment (90°C,  
211 30 min) allowed to reduce the non-spore-forming microorganisms which consume  
212 hydrogen, but with no complete elimination. Consistently, Luo et al. (2010) previously  
213 reported that heat shock treatment (90°C for 1h) had only short-term effects on hydrogen  
214 production performances. In comparison, in the case of systematic heat shock treatment, no  
215 methane was observed all along the experiments, showing the robustness of such a strategy  
216 to avoid hydrogen consumption by methanogens. Moreover, the heat shock treatment also  
217 impacted the hydrogen productivity and its stability during the successive batch reactors.  
218 Indeed, when no heat treatment was applied, low stability of hydrogen productivity was  
219 observed with a decrease of the productivity correlated with the emergence of methane. As  
220 soon as the heat shock treatment was used, as observed in the one initial heat shock  
221 experiment, the maximum hydrogen productivity was low but more stable during the 6  
222 successive batch reactors. A systematic heat shock treatment led to an increase of hydrogen  
223 productivity, reaching a stable value of  $1.51 \pm 0.29$  mLH<sub>2</sub>/gVS/h after 8 cycles of effluent  
224 recycling (*i.e* from reactor 9), value being 331 % higher than the one found in the first dark  
225 fermentation. Consistently, O-Thong et al. (2016) reported the benefit to recirculate the  
226 effluent (with a recirculation rate of 30%) from methanogenic reactor into continuous  
227 acidogenic reactor by improving the hydrogen productivity from 60 mLH<sub>2</sub>/gCOD to 188  
228 mLH<sub>2</sub>/gCOD when compared to no recirculation.

229 The hydrogen lag phase was also positively impacted by effluent recycling. For the no heat  
230 shock and the one initial heat shock treatment modes, the hydrogen lag phase was reduced  
231 by  $67.7\pm 1.6\%$  after the first cycle of effluent recycling (i.e., between reactors 1 and 2). The  
232 difference found between the one initial (25.3 h) and the systematic heat shock treatment  
233 (10.1 h) was likely due to a difference in the amount of microorganisms and the microbial  
234 activity between the used inocula, although the fermentation conditions were similar for  
235 both experiments. However, whatever the treatment used, the lag phase reached a similar  
236 value after the first effluent recycling, *i.e.*  $7.5\pm 0.5$ ,  $7.9\pm 2.0$  and  $8.8\pm 2.6$  h for no heat shock,  
237 one initial heat shock and systematic heat shock treatments, respectively. These values were  
238 lower than the ones reported by Elbeshbishy et al. (2011) using heat shock treatment ( $70^{\circ}\text{C}$ ,  
239 30 min) without effluent recycling strategy ( $16.3\pm 1.6$  h), which confirms the benefits of  
240 recycling the effluent on the reduction and the stabilization of the lag phase.

241 Thus, the combination of effluent recycling and heat shock treatment of the liquid phase for  
242 dark fermentation of OFMSW in successive batch reactors had a global positive impact on  
243 all hydrogen performance indicators, such as  $\text{H}_2$  yields, maximum productivity and lag  
244 phase, and more especially when a systematic heat shock treatment was applied.

### 245 **3.2. Macroscopic parameters and inhibitors accumulation analysis**

246 Recycling the effluent can also promote the accumulation of soluble chemicals such as  
247 VFAs, ions or ammonia which can subsequently affect the microbial activity and lead to  
248 the inhibition of hydrogen production. The main parameters related to the chemicals  
249 produced during dark fermentation were measured at the beginning (VFAs, pH,  
250 conductivity) and at the end (pH, conductivity, Total Ammonia Nitrogen (TAN)) of each  
251 reactor during the control (No), the one initial heat shock (One) and the systematic heat

252 shock (Syst) experiments (Fig. 3). The initial pH was similar for all the experiments  
253 independently of the treatment method and number of effluent recycling cycles. The  
254 average initial pH (Fig. 3A) was estimated at  $7.0\pm 0.5$ ,  $7.3\pm 0.5$  and  $7.6\pm 0.4$  in the control,  
255 the one initial heat shock and the systematic heat shock experiments, respectively. At the  
256 end of fermentation, the pH was constant and lower than the initial pH ( $5.7\pm 0.3$ ,  $5.4\pm 0.4$   
257 and  $5.5\pm 0.2$  for no heat shock, one initial heat shock and systematic heat shock  
258 respectively). The final pH was consistent with similar experiment of dark fermentation  
259 using kitchen waste as substrate (14.3 gVS/L) with a final pH measured at  $5.88\pm 0.04$  when  
260 the optimal hydrogen production was observed (Slezak et al., 2017). A high range of initial  
261 pH, ie. from 5.0 to 9.0, has been reported for hydrogen production in batch dark  
262 fermentation. Kim et al. (2011) observed a maximum for hydrogen production when the  
263 initial pH was fixed at 8.0 (followed by a drop at pH 5) using food waste as substrate. A pH  
264 lower than 5 can also affect hydrogen production as shown by Liu et al. (2006) during dark  
265 fermentation of household solid waste. Consistently, the pH measured in the present  
266 experiments was always in an optimal range for hydrogen production, decreasing from  
267  $7.6\pm 0.4$  to  $5.5\pm 0.2$  over each batch operation. As a consequence, the decrease of hydrogen  
268 performances observed in the control and the one initial heat shock method was not  
269 correlated with of the pH variation. For control and systematic heat shock treatment modes,  
270 an increase of the initial VFAs concentration was observed (Fig. 3B) from 16.2 and 44.2  
271 mgCOD/gVS in reactor 1 to 105.1 and 123.0 mgCOD/gVS in reactor 6, respectively. In the  
272 next successive batch reactors for the systematic heat shock experiment, an average and  
273 stable value of  $106\pm 15$  mgCOD/gVS was measured. In the one initial heat shock treatment  
274 experiment, the concentration of initial VFAs was always lower than in the two other

275 modes (except for reactor 5 reaching similar value at 74.8 mgCOD/gVS). In the literature,  
276 it was found that a high initial content of VFAs can be detrimental to the microbial activity  
277 through the acidification of the medium resulting in a failure of the process (Ariunbaatar et  
278 al., 2015). It was reported that the undissociated forms of these acids (acetic or butyric  
279 acids) can cross the cell membrane and acidify the cellular medium by releasing protons  
280 during his dissociation, which implies an increase of the energy demand to maintain  
281 constant the internal pH. Here, the improvement of the overall hydrogen performances in  
282 the systematic heat shock experiment (reduction of hydrogen lag phase and increase of  
283 productivity) showed that the initial VFA concentration was not detrimental to hydrogen  
284 production. As well, the inhibition of hydrogen production observed in the control and  
285 during the one initial heat shock treatment experiment was not correlated to the VFA  
286 accumulation since the concentrations found for these two conditions were close to the  
287 systematic heat shock treatment experiment. The stability of the initial VFA concentration  
288 can be easily linked to the potential of water absorption of the organic matter during  
289 fermentation. After each dark fermentation, the liquid phase (called “effluent”) was  
290 separated from the solid phase by sieving, the quantity of effluent collected allow to know  
291 the amount of liquid absorbed in the ‘fermentate’ (*i.e.* solid phase). Thus, it was measured  
292 that an average of  $51 \pm 8.3$  % the liquid phase was absorbed in the solid phase whatever the  
293 operation mode (mainly due to the presence of paper and cardboard). As a consequence,  
294 and in addition to the effluent recycled for the next dark fermenter, additional leachate has  
295 to be incorporated to maintain similar conditions along the experiments (TS of 15%),  
296 leading to a dilution and a stabilization of the VFAs concentration.

297 Ionic strength is also known to have a detrimental effect on hydrogen production, as  
298 already reported elsewhere (Paillet et al., 2019). In the present study, the conductivity,  
299 which is correlated to the total ionic strength, remained constant during the entire duration  
300 of the experiments, whatever the operation mode (control, one initial heat shock and  
301 systematic heat shock treatment), showing no accumulation of inhibitory ions in the  
302 medium (Fig. 3.C). Finally, as reported in Fig. 3.D, no accumulation of ammonia nitrogen  
303 (TAN) was detected. For the systematic and one initial heat shock treatment experiment, a  
304 similar trend was observed with average final concentrations of  $2.1\pm 0.4$  gN/L and  $2.1\pm 0.2$   
305 gN/L respectively. In the control, from reactor 1 to 3, a slight increase of TAN was  
306 observed from 2.42 to 3.1 gN/L to reach a stable phase until a drop at 2.1 gN/L for the last  
307 reactor. According to the literature, the concentrations observed in the present study were in  
308 an adequate range for hydrogen production and not associated to ammonia inhibition.  
309 Indeed, Pan et al. (2013) showed an inhibition of hydrogen production for TAN  
310 concentration higher than 3.5 gN/L. Thus, from the macroscopic parameters analysis, the  
311 main reasons for the decline of hydrogen production is not due to the accumulation of  
312 inhibitors when effluent recycling strategy is applied.

### 313 **3.3. Metabolite production**

314 Based on the initial and final concentrations of metabolites (VFAs, lactate, ethanol,  
315 butanol), the efficiency of production of metabolites in dark fermentation was estimated.  
316 The average COD of metabolites produced per fermentation was  $142.8\pm 21.4$ ,  $109.1\pm 27.3$   
317 and  $131.4\pm 27.5$  mgCOD/gVS for no heat shock, one initial heat shock and systematic heat  
318 shock treatment experiments, respectively. The metabolites produced were in the same  
319 range than the values usually found in dark fermentation of organic waste ( $140.7\pm 23$

320 mgCOD/gVS) (Ghimire et al., 2018). This suggests an efficient microbial activity in each  
321 reactor and that heat shock treatment and effluent recycling did not impact the global  
322 microbial conversion of OFMSW into metabolites. The distribution of metabolites  
323 produced during each fermentation are presented in Fig. 4 for the three treatment modes. In  
324 the control condition, the main metabolites produced during the first reactor were acetate  
325 and butyrate, representing 23.0% and 60.7% of the total COD, respectively (*i.e.* 33.5 and  
326 88.3 mgCOD/gVS, respectively). After the first effluent recycling, other metabolites were  
327 produced such as lactate (34.2 and 12.4 mgCOD/gVS for reactors 2 and 3, respectively) or  
328 caproate that gradually increased in proportion, from 6.1%<sub>COD</sub> to 32.6%<sub>COD</sub> between reactor  
329 1 and 6. The metabolite shift observed between the production of lactate and caproate can  
330 be explained by elongation chain mechanisms (Han et al., 2019). As demonstrated by Zhu  
331 et al.(2015), lactate can be used as electron donor for the synthesis of medium-chain  
332 carboxylic acids (MCCAs) as caproate under anaerobic fermentative conditions by  
333 *Clostridium* sp. The proportion of valerate also increased from 5.7%<sub>COD</sub> in reactor 4 to  
334 14.0%<sub>COD</sub> in reactor 6. Overall, the recycling strategy with no heat treatment showed a  
335 modification of the selected metabolic pathways with the emergence of lactate, caproate  
336 and valerate at the detriment of butyrate. In the one initial heat shock mode, the two main  
337 metabolites found in the first reactor were acetate and butyrate at a proportion of 37.1%<sub>COD</sub>  
338 and 49.5%<sub>COD</sub>, respectively. After the first cycle of effluent recycling, the proportions of  
339 acetate and butyrate decreased and a new metabolite, *ie.* Caproate, was detected in a  
340 significant proportion. Caproate reached  $10.2\pm 3.5$  %<sub>COD</sub> at the end of fermentation in the  
341 reactors 2, 3 and 4. Significant lactate accumulation was also observed, from 28.7%<sub>COD</sub> to  
342 57.0%<sub>COD</sub> in reactors 2 and 6, respectively. Compared to the control, lactate was



343 continuously produced after the first effluent recycling and became the main metabolite  
344 produced. Thus, one initial heat shock condition likely stabilized the metabolic pathways by  
345 promoting the production of lactate. In the systematic heat shock treatment mode, the main  
346 metabolite was butyrate, with a proportion ranging from 26.4%<sub>COD</sub> to 63.5%<sub>COD</sub>. Acetate  
347 and ethanol (except in reactor 11) were also produced constantly ( $10.6 \pm 2.7$  %<sub>COD</sub> and  
348  $10.1 \pm 5.6$  %<sub>COD</sub> respectively). The other metabolites such as propionate, caproate, lactate  
349 and butanol, were produced with higher variability. Butanol appeared in 8 reactors out of  
350 16 and lactate in 12 out of 16 with a high standard deviation (*i.e.*  $16.2 \pm 11.0$  %<sub>COD</sub>) showing  
351 the instability in the production of these metabolites in the systematic heat shock treatment  
352 condition. In dark fermentation, the two main metabolic pathways producing hydrogen are  
353 the acetate and butyrate pathways and these two metabolites are the main end products  
354 (Cavinato et al., 2012). Consistently, in the present study, the best hydrogen yields  
355 observed during no heat shock and one initial heat shock conditions ( $18.8$  mLH<sub>2</sub>/gVS and  
356  $10.5$  mLH<sub>2</sub>/gVS, respectively) were obtained in the first reactor, when the overall  
357 proportion of acetate and butyrate represented 83.6%<sub>COD</sub> and 86.6%<sub>COD</sub> of the total  
358 metabolites, respectively. Interestingly, high concentration of butyric acid can inhibit the  
359 activity of methanogens as already discussed by Li et al., (2018), which could be another  
360 explanation of the delay observed for the development of methanogens. In the systematic  
361 heat shock treatment condition, the accumulation of these two metabolites and especially  
362 butyrate was in line with the constant production of hydrogen. In addition, the butyrate to  
363 acetate ratio (B/A) can reflect the theoretical hydrogen yield. In particular, with a ratio B/A  
364 of 1.5, it was suggested a hydrogen yield of  $2.5$  molH<sub>2</sub>/mol<sub>hexose</sub> (Hawkes et al., 2007). In  
365 this study, the B/A ratio was  $1.48 \pm 0.51$  and  $1.47 \pm 0.54$  for no heat shock and systematic

366 heat shock treatment conditions, respectively, while for one initial heat shock treatment, the  
367 ratio was lower ( $0.80\pm 0.30$ ) mainly caused by the decrease of butyrate production.

368 Several metabolic pathways enter also in competition with hydrogen production since they  
369 do not generate hydrogen during the conversion of organic matter which is an additional  
370 reason of hydrogen inhibition. For instance, propionate, caproate and lactate pathways are  
371 the mostly found non-hydrogen producing pathways (Motte et al., 2013). Thus, the  
372 decrease of butyrate, correlated with the emergence of valerate, caproate and lactate, is an  
373 additional explanation for the low production of hydrogen observed in no heat shock and  
374 one initial heat shock conditions. However, regarding the B/A ratio of these two modes, the  
375 low production of hydrogen during the no heat shock experiment was mainly caused by  
376 hydrogenotrophic methanogens since the B/A ratio ( $1.48\pm 0.51$ ) was closed to the optimal  
377 value. Whereas, in one initial heat shock condition, the lower ratio of B/A ( $0.80\pm 0.30$ )  
378 showed a stronger selection of competitive metabolic pathways of hydrogen production,  
379 especially lactate. Thus, one initial heat shock condition could be a short-term solution to  
380 avoid hydrogen consumption through methanogenesis but the effluent recycling strategy  
381 seems to promote the competitive metabolic pathways of hydrogen production.

382 Interestingly, ethanol and butanol were also produced in significant proportions during  
383 systematic heat shock treatment mode ( $10.6\pm 2.7$  %COD and  $10.1\pm 5.6$  %COD,  
384 respectively). Xue & Cheng (2019) showed that *Clostridium* sp., which are the major  
385 sporulating hydrogen-producing bacteria, can also produce butanol and ethanol via ABE  
386 (acetone-butanol-ethanol) fermentation, by solventogenesis. The solventogenic pathway  
387 refers to the assimilation of acids to produce solvents. To better understand the impact of  
388 these metabolites on the overall process, a principal component analysis (PCA) was

389 performed (Fig. 5) and shows that hydrogen production was negatively correlated to  
390 ethanol and butanol production suggesting that these two molecules were not involved in  
391 hydrogen production pathways. This is consistent with the study of Kim et al. (2011) who  
392 showed that, when the environmental conditions are favorable for hydrogenases, oxidized  
393 by-products such as acetate and butyrate, are concomitantly produced with hydrogen. In  
394 contrast, when hydrogenases are inhibited the production of ethanol and butanol pathways  
395 are favored. As reported in the literature, high partial pressure of hydrogen is a cause of  
396 changes in metabolic pathways towards the production of lactate, acetone, butanol and  
397 ethanol (Levin, 2004). As already discussed above, inoculum pretreatment could also  
398 negatively affect the production of hydrogen by generating stressful conditions for the  
399 microbiota. Finally, the consumption of hydrogen by homoacetogenesis (4 moles of  
400 hydrogen to produce 1 mole of acetate) can be one of the main factors of hydrogen  
401 consumption resulting in the production of acetate (Saady, 2013). Fig. 5 shows a negative  
402 correlation between acetate and hydrogen production which suggests that  
403 homoacetogenesis could have occurred during the experiment. However, no increase of  
404 acetate proportion within the produced metabolites was observed, suggesting that the  
405 effluent recycling strategy did not favor the development of homoacetogenic bacteria.  
406 Consistently, systematic heat shock treatment coupled to effluent recycling likely favored a  
407 continuous selection of microbial populations carrying metabolic pathways related to  
408 hydrogen production (mainly butyrate) and avoided hydrogen consumption by  
409 methanogens. The emergence of alternative metabolic pathways (butanol, ethanol, lactate)  
410 was probably a sign of microbial community stress.

### 3.4. Development of the fermentative microbial communities during effluent recycling for successive batch reactors

The microbial community at the beginning of each batch reactor corresponded to the microbial community of the heat pretreated inoculum (mixture of leachate and recycling effluent after the first reactor), whereas it corresponded to the fermented medium at the end of each batch reactor. Analysis of the microbial community was performed in samples collected at the beginning (7 samples) and at the end (13 samples) of the successive batch reactors, operated with systematic heat shock treatment. Considering all the samples, a total of 1082 operating taxonomic units (OTUs) were found. The most dominant taxonomic orders at start of the reactors (Fig. 6 A) were affiliated to *Pseudomonadales* (with an abundance ranging from 3% to 46% of the total sequences), *Clostridiales* (abundance from 5% to 51%), *Lactobacillales* (6 to 25%) and *Bacillales* (0 to 33%). Higher diversity was found at start of the first reactors (reactor 1 to 7) with an important proportion of *Pseudomonadales* and *Bacillales*. A shift of the microbial community was then observed, with the dominance of *Clostridiales* and *Lactobacillales* showing good stability on the microbial community from reactor 8 to 16. The samples collected at the end of fermentation showed a very stable community structure all along the experiment with two dominant taxonomic orders, *Clostridiales* and *Lactobacillales*, with relative abundances ranging from 37 to 63% and from 5% to 60%, respectively (Fig. 6B). Only in the first reactor, the presence of *Bacillales* (24% of relative abundance) was observed. Then, the *Clostridiales* maintained his population compared to the initial leachate composition while the proportion of *Lactobacillales*, initially present at 6.8%, increased largely and outcompeted the other species. The systematic decrease of microbial diversity identified at

434 the end of fermentation is consistent with previous observations of Yang & Wang, (2019)  
435 who showed a similar microbial diversity decrease during dark fermentation with only four  
436 different dominant genera after fermentation : *Clostridium sp.*, *Paraclostridium*,  
437 *Romboustia* and *Paeniclostridium*. By performing a BLASTn search using the NCBI  
438 database, at the end of fermentation, the most abundant OTUs issued from *Clostridiales*  
439 (37±8% of total relative abundance) could be affiliated to two species with close similarity  
440 (97% 16S rRNA sequence similarity): *Clostridium butyricum* or *Clostridium beijerinckii*.  
441 These two species have been widely described as efficient hydrogen-producing bacteria.  
442 Liu et al. (2011) reported a high hydrogen production with *Clostridium butyricum* (1.77  
443 mmol/mmol<sub>glucose</sub>) and *Clostridium beijerinckii* (1.72 mmol/mmol<sub>glucose</sub>) in pure cultures.  
444 *Clostridium butyricum* produces mainly butyrate which can explain the high butyrate  
445 production found in the systematic heat shock treatment condition (Cheng et al., 2011).  
446 Concerning the *Lactobacillales* order, the diversity was mainly represented by three main  
447 species. The most abundant one (16±11%) was affiliated to *Lactobacillus mucosae* or  
448 *Lactobacillus spicheri* (98% 16S rRNA sequence similarity). These species were already  
449 described as lactate producers (Meroth et al., 2004). The two other species had a slightly  
450 lower relative abundance of 9±8% and 5±4%, and were affiliated to *Lactobacillus*  
451 *plantarum* (100% rRNA sequence similarity) and *Lactobacillus paracasei* or *Lactobacillus*  
452 *casei* (100% rRNA sequence similarity), respectively, also known as lactate producers  
453 (Chen et al., 2020; Kuo et al., 2015). The lactate producers are well known to outcompete  
454 hydrogen producing bacteria. Interestingly, in the present experiment, an equilibrium was  
455 observed between *Lactobacillales* and *Clostridiales*. Some species of *Clostridium sp.* such  
456 as *C. beijerinckii* and *C. butyricum* are able to consume lactate and acetate to produce

457 butyrate and hydrogen (García-Depraect et al., 2019). Interestingly, these two species were  
458 found after fermentation with a high proportion of butyrate. Moreover, lactate  
459 concentration showed a high variability (*i.e.*  $16.2 \pm 11.0$  %<sub>COD</sub>) even though *Lactobacillales*  
460 were abundant. All this suggests that lactate was partially consumed along the fermentation  
461 process. From the metabolite distribution (Fig. 4), both the production of butyrate and  
462 lactate was highly variable. In particular, when a high proportion of lactate was detected, a  
463 low proportion of butyrate was observed and vice-versa, supporting the probable link  
464 between these two pathways. Consistently, the PCA (Fig. 5) analysis showed that lactate  
465 negatively correlated to butyrate, corroborating to this assumption. Due to the fact that  
466 lactate is involved in a no H<sub>2</sub> producing (product) or a producing hydrogen pathways  
467 (substrate), no clear correlation was observed with hydrogen. Since it influenced the  
468 statistical test, butyrate did not clearly positively correlate to hydrogen production.

#### 469 **4. Conclusion**

470 The stability of hydrogen production in a dark fermentation reactor using different effluent  
471 recycling strategies was investigated. Optimal performances were obtained when heat  
472 shock treatment was systematically applied on the recycled effluent, at the beginning of  
473 each fermentation. Indeed, by recycling the effluent a reduction of the lag phase was  
474 observed and the use of systematic heat shock treatment enhanced the hydrogen  
475 productivity and stabilized the hydrogen yield. In this study, relevant operating conditions  
476 are proposed to sustain robust and constant hydrogen production from the complex  
477 OFMSW, as a new opportunity for producing green hydrogen at larger scale.

#### 478 **References**

- 479 1. Alibardi, L., Cossu, R., 2015. Composition variability of the organic fraction of  
480 municipal solid waste and effects on hydrogen and methane production potentials. *Waste*  
481 *Manag.* 36, 147–155.
- 482 2. Ariunbaatar, J., Scotto Di Pert, E., Panico, A., Frunzo, L., Esposito, G., Lens, P.N.L.,  
483 Pirozzi, F., 2015. Effect of ammoniacal nitrogen on one-stage and two-stage anaerobic  
484 digestion of food waste. *Waste Manag.* 38, 388–398.
- 485 3. Campuzano, R., González-Martínez, S., 2016. Characteristics of the organic fraction of  
486 municipal solid waste and methane production: A review. *Waste Manag.* 54, 3–12.
- 487 4. Cavinato, C., Giuliano, a., Bolzonella, D., Pavan, P., Cecchi, F., 2012. Bio-hythane  
488 production from food waste by dark fermentation coupled with anaerobic digestion process:  
489 A long-term pilot scale experience. *Int. J. Hydrogen Energy* 37, 11549–11555.
- 490 5. Chen, P.-T., Hong, Z.-S., Cheng, C.-L., Ng, I.-S., Lo, Y.-C., Nagarajan, D., Chang, J.-S.,  
491 2020. Exploring fermentation strategies for enhanced lactic acid production with polyvinyl  
492 alcohol-immobilized *Lactobacillus plantarum* 23 using microalgae as feedstock.  
493 *Bioresour.Technol.* 308, 123266.
- 494 6. Cheng, C.-H., Hsu, S.-C., Wu, C.-H., Chang, P.-W., Lin, C.-Y., Hung, C.-H., 2011.  
495 Quantitative analysis of microorganism composition in a pilot-scale fermentative  
496 biohydrogen production system. *Int. J. Hydrogen Energy* 36, 14153–14161.
- 497 7. Dawood, F., Anda, M., Shafiullah, G.M., 2020. Hydrogen production for energy: An  
498 overview. *Int. J. Hydrogen Energy* 45, 3847–3869.
- 499 8. Elbeshbishy, E., Hafez, H., Dhar, B.R., Nakhla, G., 2011. Single and combined effect of  
500 various pretreatment methods for biohydrogen production from food waste. *Int. J.*  
501 *Hydrogen Energy* 36, 11379–11387.

- 502 9. Favaro, L., Alibardi, L., Lavagnolo, M.C., Casella, S., Basaglia, M., 2013. Effects of  
503 inoculum and indigenous microflora on hydrogen production from the organic fraction of  
504 municipal solid waste. *Int. J. Hydrogen Energy* 38, 11774–11779.
- 505 10. García-Depraect, O., Valdez-Vázquez, I., Rene, E.R., Gómez-Romero, J., López-López,  
506 A., León-Becerril, E., 2019. Lactate- and acetate-based biohydrogen production through  
507 dark co-fermentation of tequila vinasse and nixtamalization wastewater: Metabolic and  
508 microbial community dynamics. *Bioresour. Technol.* 282, 236–244.
- 509 11. Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G.,  
510 2015. A review on dark fermentative biohydrogen production from organic biomass:  
511 Process parameters and use of by-products. *Applied Energy* 144, 73–95.
- 512 12. Ghimire, A., Trably, E., Frunzo, L., Pirozzi, F., Lens, P.N.L., Esposito, G., Cazier,  
513 E.A., Escudié, R., 2018. Effect of total solids content on biohydrogen production and lactic  
514 acid accumulation during dark fermentation of organic waste biomass. *Bioresour. Technol.*  
515 248, 180–186.
- 516 13. Han, W., He, P., Shao, L., Lü, F., 2019. Road to full bioconversion of biowaste to  
517 biochemicals centering on chain elongation: A mini review. *J. Environ. Sciences* 86, 50–64.
- 518 14. Hans, M., Kumar, S., 2019. Biohythane production in two-stage anaerobic digestion  
519 system. *Int. J. Hydrogen Energy* 44, 17363–17380.
- 520 15. Hawkes, F., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D., 2007. Continuous dark  
521 fermentative hydrogen production by mesophilic microflora: Principles and progress. *Int. J.*  
522 *Hydrogen Energy* 32, 172–184.
- 523 16. Jang, S., Kim, D.-H., Yun, Y.-M., Lee, M.-K., Moon, C., Kang, W.-S., Kwak, S.-S.,  
524 Kim, M.-S., 2015. Hydrogen fermentation of food waste by alkali-shock pretreatment:



525 Microbial community analysis and limitation of continuous operation. *Bioresour. Technol.*  
526 186, 215–222.

527 17. Kim, D.H., Kim, S.H., Jung, K.W., Kim, M.S., Shin, H.S., 2011. Effect of initial pH  
528 independent of operational pH on hydrogen fermentation of food waste. *Bioresour.*  
529 *Technol.* 102, 8646–8652.

530 18. Kuo, Y.-C., Yuan, S.-F., Wang, C.-A., Huang, Y.-J., Guo, G.-L., Hwang, W.-S., 2015.  
531 Production of optically pure l -lactic acid from lignocellulosic hydrolysate by using a newly  
532 isolated and d -lactate dehydrogenase gene-deficient *Lactobacillus paracasei* strain.  
533 *Bioresour. Technol.* 198, 651–657.

534 19. Levin, D., 2004. Biohydrogen production: prospects and limitations to practical  
535 application. *Int. J. Hydrogen Energy* 29, 173–185.

536 20. Li, Z., Chen, Z., Ye, H., Wang, Y., Luo, W., Chang, J.-S., Li, Q., He, N., 2018.  
537 Anaerobic co-digestion of sewage sludge and food waste for hydrogen and VFA production  
538 with microbial community analysis. *Waste Manag.* 78, 789–799.

539 21. Liu, Dawei, Liu, Dapeng, Zeng, R.J., Angelidaki, I., 2006. Hydrogen and methane  
540 production from household solid waste in the two-stage fermentation process. *Water*  
541 *research* 40, 2230–2236.

542 22. Liu, I.-C., Whang, L.-M., Ren, W.-J., Lin, P.-Y., 2011. The effect of pH on the  
543 production of biohydrogen by clostridia: Thermodynamic and metabolic considerations.  
544 *Int. J. Hydrogen Energy* 36, 439–449.

545 23. Luo, G., Xie, L., Zou, Z., Wang, W., Zhou, Q., 2010. Evaluation of pretreatment  
546 methods on mixed inoculum for both batch and continuous thermophilic biohydrogen  
547 production from cassava stillage. *Bioresour. Technol.* 101, 959–964.

548 24. Meena, R.A.A., Rajesh Banu, J., Yukesh Kannah, R., Yogalakshmi, K.N., Kumar, G.,  
549 2020. Biohythane production from food processing wastes – Challenges and perspectives.  
550 *Bioresour. Technol.* 298, 122449.

551 25. Meroth, C.B., Hammes, W.P., Hertel, C., 2004. Characterisation of the Microbiota of  
552 Rice Sourdoughs and Description of *Lactobacillus spicheri* sp. nov. *Syst. Appl.*  
553 *Microbiology* 27, 151–159.

554 26. Motte, J.-C., Trably, Eric., Escudié, Renaud., Hamelin, J., Steyer, J.-P., Bernet, N.,  
555 Delgenes, J.-P., Dumas, C., 2013. Total solids content: a key parameter of metabolic  
556 pathways in dry anaerobic digestion. *Biotech. for biofuels* 6, 164.

557 27. O-Thong, S., Suksong, W., Promnuan, K., Thipmune, M., Mamimin, C., Prasertsan,  
558 P., 2016. Two-stage thermophilic fermentation and mesophilic methanogenic process for  
559 biohythane production from palm oil mill effluent with methanogenic effluent recirculation  
560 for pH control. *Int. J. Hydrogen Energy* 41, 21702–21712.

561 28. Paillet, F., Barrau, C., Escudié, R., Trably, E., 2019. Inhibition by the ionic strength of  
562 hydrogen production from the organic fraction of municipal solid waste. *Int. J. Hydrogen*  
563 *Energy* 45. 5854-5863

564 29. Pan, J., Chen, X., Sheng, K., Yu, Y., Zhang, C., Ying, Y., 2013. Effect of ammonia on  
565 biohydrogen production from food waste via anaerobic fermentation. *Int. J. Hydrogen*  
566 *Energy* 38. 12747–12754.

567 30. Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed  
568 cultures dark fermentation: Unresolved challenge. *Int. J. Hydrogen Energy* 38, 13172–  
569 13191.

570 31. Slezak, R., Grzelak, J., Krzystek, L., Ledakowicz, S., 2017. The effect of initial organic  
571 load of the kitchen waste on the production of VFA and H<sub>2</sub> in dark fermentation. *Waste*  
572 *Manag.* 68, 610–617.

573 32. Xu, R., Xu, S., Florentino, A.P., Zhang, L., Yang, Z., Liu, Y., 2019. Enhancing  
574 blackwater methane production by enriching hydrogenotrophic methanogens through  
575 hydrogen supplementation. *Bioresour. Technol.* 278, 481–485.

576 33. Xue, C., Cheng, C., 2019. Butanol production by *Clostridium*. *Adv. in Bioenergy* 4, 35–  
577 77.

578 34. Yang, G., Wang, J., 2019. Changes in microbial community structure during dark  
579 fermentative hydrogen production. *Int. J. Hydrogen Energy* 44, 25542–25550.

580 35. Zhang, B., He, P.J., Lü, F., Shao, L.M., Wang, P., 2007. Extracellular enzyme activities  
581 during regulated hydrolysis of high-solid organic wastes. *Water Research* 41, 4468–4478.

582 36. Zhu, H., Béland, M., 2006. Evaluation of alternative methods of preparing hydrogen  
583 producing seeds from digested wastewater sludge. *Int. J. Hydrogen Energy* 31, 1980–1988.

584 37. Zhu, X., Tao, Y., Liang, C., Li, X., Wei, N., Zhang, W., Zhou, Y., Yang, Y., Bo, T.,  
585 2015. The synthesis of n-caproate from lactate: a new efficient process for medium-chain  
586 carboxylates production. *Sci Rep* 5, 14360.

587

588

589

590

591

592 **Fig. 1** : Schematic representation of the three different conditions (heat treatment and  
593 effluent recirculation) used for successive batch dark fermenters.

594 **Fig. 2** : Hydrogen production performances (maximum yield ■, maximum productivity ■  
595 and lag phase •) from dark fermentation of OFMSW using different heat shock treatment  
596 conditions (no heat shock, one initial heat shock and systematic heat shock).

597 **Fig. 3** : Evolution of pH, total volatile fatty acids concentration (VFAs), conductivity and  
598 Total Ammonia Nitrogen (TAN) for the three heat shock treatment conditions (no heat  
599 shock – No; one initial heat shock - One; systematic heat shock -Syst).

600 **Fig. 4** : Metabolite distribution and total COD production (gCOD/gVS) in dark  
601 fermentation batch reactors using different heat shock treatment conditions (no heat shock,  
602 one-time heat shock and systematic heat shock).

603 **Fig. 5** : PCA of the performances of effluent recirculation reactors for systematic heat  
604 shock treatment, H<sub>2</sub>: hydrogen yield (mLH<sub>2</sub>/gVS).

605 **Fig. 6** : Relative abundance of microbial communities at the start (A) and at the end (B) of  
606 dark fermentations during systematic heat shock condition.

607 **Table 1** : Composition of the freshly prepared OFMSW. Data are reported as percentage on  
608 wet weight basis (% w/w)

609 **Table 2** : Time to reach the maximum of hydrogen productivity (in h) and the time of first  
610 significant methane detection (in h) during the three experiments (no heat shock, one initial  
611 heat shock and systematic heat shock)

612

613

614

615

616

617

618

619

620

Category	Elements	% w/w	TS (gTS/g)	VS (gVS/g)
Food waste	Meat	7.0	0.45±0.01	0.43±0.01
	Coffee grounds	3.9	0.32±0.01	0.32±0.01
	Rice	4.3	0.28±0.01	0.27±0.01
	Potatoes	20.9	0.26±0.01	0.24±0.01
	Bread	5.1	0.96±0.01	0.95±0.01
	Yogurt	2.0	0.13±0.01	0.12±0.01
Garden waste	Grass	5.0	0.28±0.01	0.25±0.01
Paper	Office paper	31.7	0.94±0.01	0.76±0.01
Cardboard	Moving cardboard	16.7	0.92±0.01	0.77±0.01
	Paper folder	3.4	0.94±0.01	0.91±0.02

621 **Table 1**

622

623

624

625

626

627

	Reactor N°	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
No heat shock	tH <sub>2</sub> max (h)	29.7	22.8	15.1	14.7	12.0	20.1										
	tCH <sub>4</sub> (h)	50.7	79.8	42.1	17.7	12.0	8.1										
One initial heat shock	tH <sub>2</sub> max (h)	31.9	16.0	12.0	16.0	16.0	16.0										
	tCH <sub>4</sub> (h)	127.9	23.3	16.0	16.0	12.0	12.0										
Systematic heat shock	tH <sub>2</sub> max (h)	26.0	16.6	10.0	14.1	15.0	15.0	18.0	24.0	21.1	12.7	15.8	12.0	13.9	12.0	24.1	48.0
	tCH <sub>4</sub> (h)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

628 **Table 2**

629

630

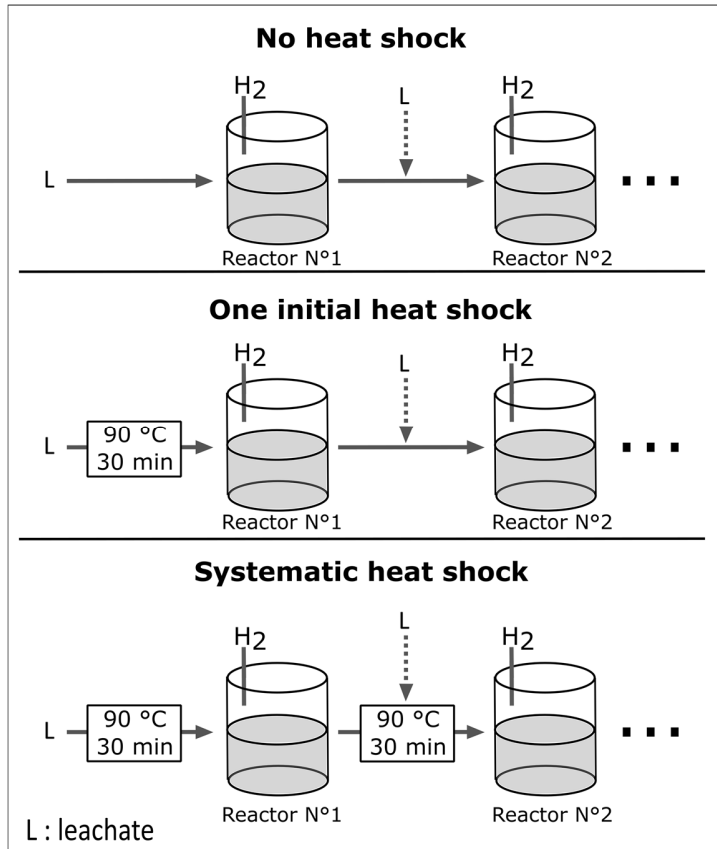
631

632

633

634

635 **Fig. 1.**



636  
637  
638  
639  
640  
641  
642  
643  
644  
645  
646  
647  
648  
649  
650  
651  
652

Fig. 2.

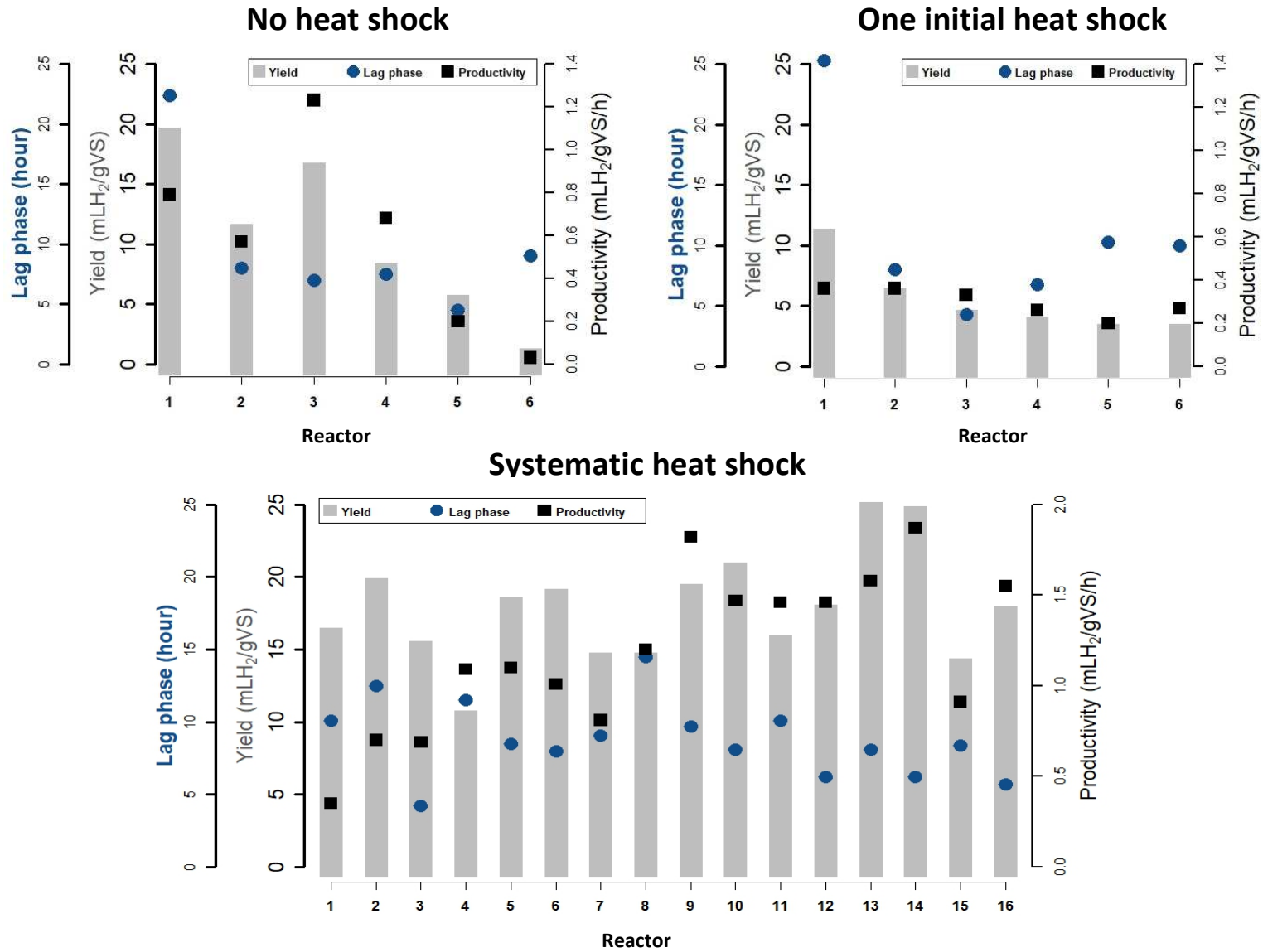




Fig. 3.

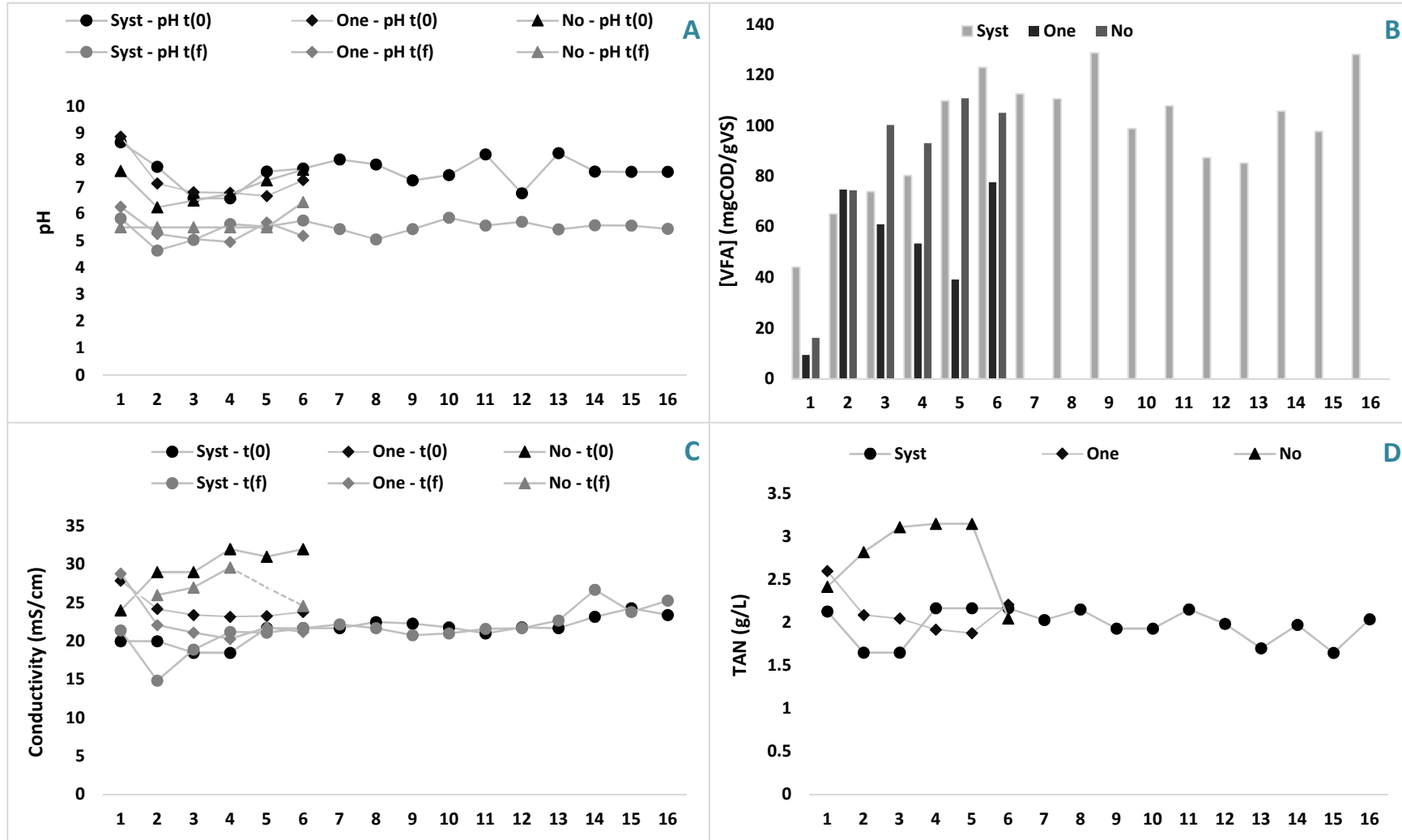
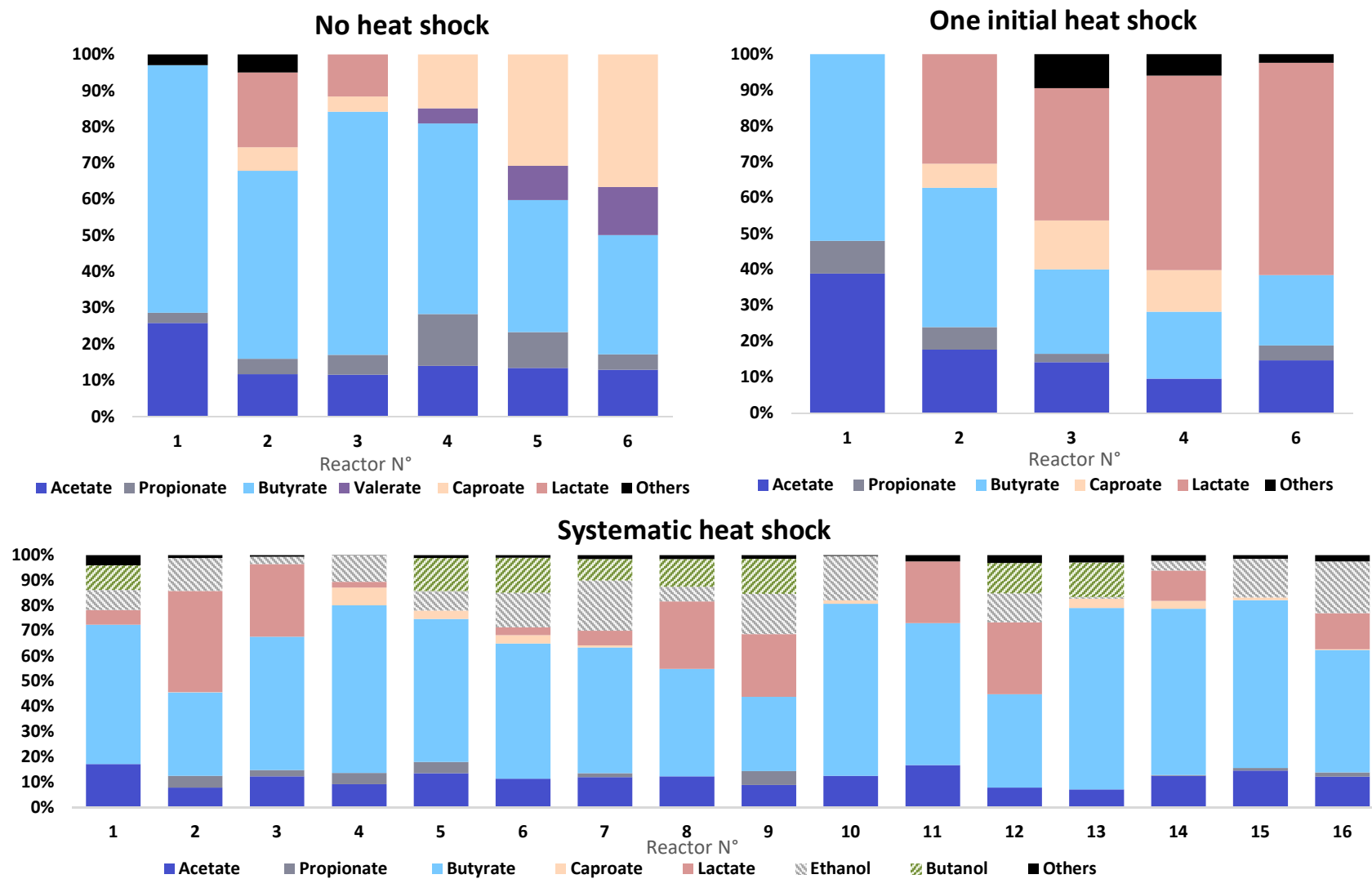
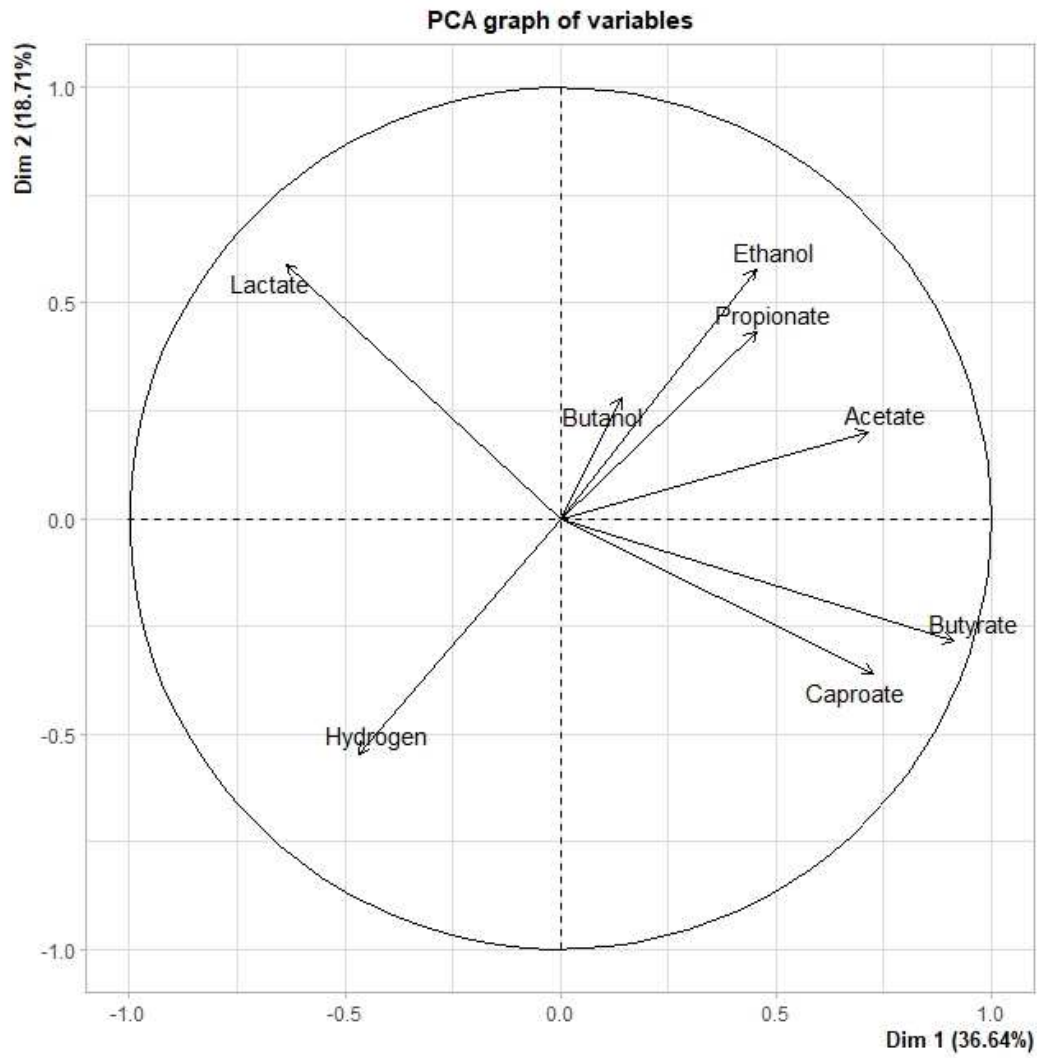


Fig. 4.



1 Fig. 5.



2

3

4

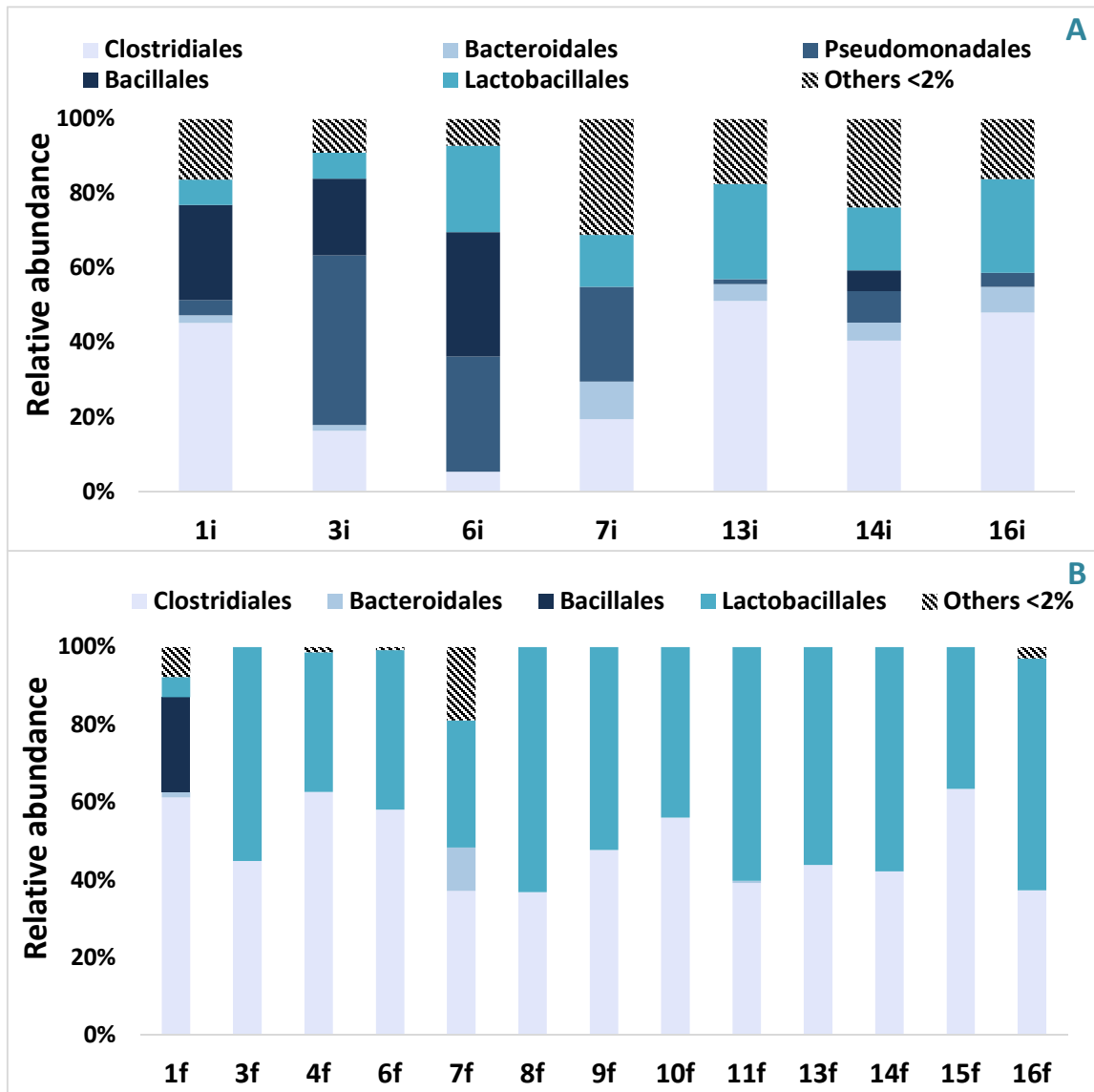
5

6

7

8

9 Fig. 6.



10  
11  
12  
13  
14