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## Co-digestion of wastewater sludge: choosing the optimal blend

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1 **Title**

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3

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19 **Declarations of interest:** none

20

21 **Abstract**

22 Anaerobic co-digestion (AcoD) is a promising strategy to increase the methane production of  
23 anaerobic digestion plants treating wastewater sludge (WAS). In this work the degradability  
24 of six different mixtures of WAS with fish waste (FW) or garden-grass (GG) was evaluated  
25 and compared to the three mono-digestions. Degradation performances and methanogenic

26 pathways, determined with the isotopic signatures of biogas, were compared across time. Fish  
27 and grass mono-digestion provided a higher final methane production than WAS mono-  
28 digestion. In co-digestion the addition of 25 % of fish was enough to increase the final  
29 methane production from WAS while 50 % of grass was necessary. To determine the optimal  
30 blend of WAS co-digestion two indicators were specifically designed, representing the  
31 maximum potential production (ODI) and the expected production in mono-digestion  
32 conditions (MDI). The comparison between these indicators and the experimental results  
33 showed that the most productive blend was composed of 75% of co-substrate, fish or grass,  
34 with WAS. Indeed, the final methane production was increased by 1.9 times with fish and by  
35 1.7 times with grass associated to an increase of the methane production rate by 1.5 times.  
36 Even if the same succession of methanogenic pathways across time was observed for the  
37 different mixtures, their relative proportions were different. Sewage sludge degradation was  
38 mostly achieved through hydrogenotrophic pathway as confirmed by the archaeal analysis  
39 while acetoclastic archaea were identified for fish and grass degradation.

40

#### 41 **Keywords**

42 Anaerobic digestion - methanogenic pathways – grass – fish – carbon-isotopic fractionation

43

#### 44 1. Introduction

45 Anaerobic Digestion (AD) is a multistep biological process allowing to convert various  
46 types of organic waste into a renewable energy, the biogas (composed of CH<sub>4</sub> and CO<sub>2</sub>) and  
47 digestate. This bioprocess has been used for more than a century, in particular to stabilise the  
48 wastewater sludge (WAS) obtained from wastewater treatment plants (Astals et al., 2012). It  
49 is particularly attractive as it allows to simultaneously produce energy in the form of biogas  
50 and to reduce the volume of sludge (Luostarinen et al., 2009). However, the benefit of using

51 only WAS to produce methane by AD is limited by its low C/N ratio and low digestion  
52 efficiency, leading to low CH<sub>4</sub> production yield (Astals et al., 2013; Park et al., 2016).

53 A way to overcome this major drawback is to balance the low C/N ratio by mixing  
54 wastewater sludge with other substrates richer in carbon. This strategy of mixing different  
55 types of substrates, or performing anaerobic co-digestion (AcoD), has multiple advantages as:  
56 1) improving the performances of digesters treating wastewater sludge (Mata-Alvarez et al.,  
57 2014), 2) treating several types of waste at the same time and 3) limiting the risk of inhibition  
58 that can occur during mono-digestion with the production of inhibitors for example (Borowski  
59 and Kubacki, 2015). Almost any type of organic waste can be treated by AD but each of them  
60 has specific properties which can bring some advantages and disadvantages. For example,  
61 animal manure and slaughterhouse waste have a high organic content but usually cause  
62 process disturbances in mono-digestion because of their high proteins and/or lipids content.  
63 This can lead to the accumulation of ammonia, volatile fatty acids (VFA) and long chain fatty  
64 acids (LCFA) (Hansen et al. 1998; Pitk et al. 2013, Borowski & Kubacki 2015) known to be  
65 responsible of process failure. WAS as a co-substrate for these waste allows to dilute  
66 compounds potentially leading to inhibition, such as proteins and lipids and limit the risk of  
67 inhibition by a too fast acidification thanks to the high buffer capacity of the WAS (Prabhu  
68 and Mutnuri, 2016).

69 Different waste have already been successfully tested to improve WAS anaerobic  
70 digestion. In association with slaughterhouse waste, Borowski & Kubacki (2015) succeeded  
71 in increasing the specific methane production by 2-fold when 50 % of slaughterhouse waste  
72 was mixed to WAS at an organic loading rate (OLR) of 4 kgVS/m<sup>3</sup>d. Neither ammonia nor  
73 LCFA inhibition was observed and an effect of VFA accumulation was observed only when  
74 the OLR was superior to 4 kgVS/m<sup>3</sup>d. Wickham et al. (2016) tested several waste such as  
75 food waste, paper pulp, fat-grease-oil (FOG) waste and dehydrated *Ulva* macroalgae. Each

76 substrate was mixed at different ratio with WAS (5, 10 and 15 % by weight). Final methane  
77 production was increased by three to six times thanks to the co-digestion compared to mono-  
78 digestion of WAS.

79 In this study, different mixtures of wastewater sludge (WAS) with garden-grass (GG) or  
80 fish waste (FW) as co-substrates were tested to determine the optimal blend allowing for the  
81 most efficient CH<sub>4</sub> production. Total fish production in the world has expanded since the last  
82 five decades from 20 million tons in 1960 to 167.2 million tons in 2014 mainly due to the  
83 increase of the aquaculture production (FAO, 2016). The amount of waste provided by the  
84 fishery industries (as canneries) is important. Some authors evaluated the possibility to use  
85 different parts of the fish (skins, viscera, bones ...) (Donoso-Bravo et al., 2015), or different  
86 fish species (Eiroa et al., 2012) for methane production with high biodegradability level.  
87 However, this type of waste can be rich in protein and/or lipids inducing ammonia and/or  
88 LCFA inhibition as observed by Eiroa et al. (2012). Literature on the possibility to use fish  
89 waste as co-substrate in AD is still scarce and to the best of our knowledge co-digestion of  
90 fish waste with wastewater sludge was not studied yet.

91 Grass has a high potential as renewable biomass source due to its high biodegradability  
92 and biogas production potential (Dai et al., 2016; Prochnow et al., 2009). Using grass as a  
93 feedstock can lead to an ammonia accumulation due to the high protein content of certain  
94 types of green waste (Prochnow et al., 2009; Ward et al., 2008). Several investigations were  
95 conducted on the anaerobic co-digestion (AcoD) of WAS and grass and showed an  
96 enhancement of methane content (Dai et al., 2016; Hidaka et al., 2013). Nonetheless further  
97 studies are needed to understand the effect of the grass addition in order to optimise the  
98 methane production.

99 The aim of this study is to investigate the possibility for improving wastewater sludge  
100 degradation during anaerobic co-digestion with fish waste or garden grass. Degradation

101 performances and methanogenic pathway, determined with the isotopic signatures of biogas  
102 (Conrad, 2005), were compared across time. Two biodegradation indicators were specifically  
103 designed to determine the mixes enabled to improve the methane production. As far we know  
104 the impact of the anaerobic co-digestion on the methanogenic pathway monitored by the  
105 isotopic analysis has not been studied yet.

106

## 107 2. Methods

### 108 2.1. Feedstock preparation and characterisation

109 Wastewater sludge came from an industrial wastewater treatment plant (Valenton,  
110 France). Two organic co-substrates were tested. Fish waste was collected from a fish shop and  
111 grass from the mowing of the Institute's lawn. Both waste were crushed and the solid part was  
112 stored at 4°C during two days before they were used.

113 The inoculum came from a mesophilic full scale anaerobic digester treating primary  
114 sludge at the Valenton (France) wastewater treatment plant. In order to degrade the residual  
115 organic matter in excess it was stored at 35°C during two weeks in anaerobic condition  
116 without feeding before being used.

117 All substrates and inoculum were characterised by different chemical analyses and the  
118 results are summarised in the Table 1.

### 119 2.2. Co-digestion experimental set-up

120 In total 27 anaerobic batch bioreactors were set-up using 1 L glass bottles (700 mL  
121 working volume). Each digester was inoculated with methanogenic sludge and fed with a  
122 mixture of a main substrate (wastewater sludge) and one co-substrate (fish waste or grass) to  
123 reach a substrate/inoculum ratio of 12 gCOD/ 1.2 gCOD. Different ratios of main substrate  
124 /co-substrate were tested (25/75, 50/50, 75/25) as detailed in the supplementary Table A.1.  
125 Controls with 100 % of wastewater sludge, fish waste or grass were also carried out. All the

126 digesters were complemented with a biochemical potential buffer (International Standard ISO  
127 11734 (1995)) to reach a final working volume of 700 mL. All incubations were performed in  
128 triplicate. The bioreactors were then sealed with a screw cap and a rubber septum. The  
129 headspaces were flushed with N<sub>2</sub> (purity > 99.99 %, Linde gas SA) and the bottles were  
130 incubated at 35°C in the dark and without agitation.

131 Weekly, 6 mL of liquid phase were sampled through the septum using a syringe and  
132 centrifuged at 10,000 g for 10 minutes. The supernatant and the pellet were snap frozen and  
133 kept at -20°C for chemical analysis and -80°C for microbial analysis.

### 134 2.3. Biochemical Methane Potential (BMP) experimental setup

135 BMP tests were carried out for each substrate in mono-digestion to assess their methane  
136 maximal production under optimal parameters. The ratio substrate/inoculum used in BMP test  
137 was 0.7 gCOD/7 gCOD to limit the latency due to the microbial growth. The biochemical  
138 potential buffer previously mentioned was used to reach a final volume of 500 mL in 1L glass  
139 bottles. As for the batch experiment, bottles were sealed, flushed with N<sub>2</sub> and incubated at  
140 35°C in the dark without agitation. The experiment was made in triplicate. Gas production  
141 and composition were followed over time. A control containing only the inoculum was  
142 carried out in parallel and the biogas production of this control was taken into account to  
143 calculate the substrates gas productions. The mixtures details are presented in the  
144 supplementary Table A.1.

### 145 2.4. Gas production and stable carbon isotope signature

146 The biogas accumulation in the headspace was measured using a differential manometer  
147 (Digitron 2082P). The biogas was then analysed directly in the headspace using a micro gas  
148 chromatograph (CP4900, Varian) as described in Chapleur et al. (2014). Data were used to  
149 calculate the biogas production at standard temperature and pressure. Different parameters

150 used to quantify the methane production potential were calculated using R CRAN software  
151 and the Gompertz equation with Grofit package as described in Poirier et al. (2016):

$$y(t) = A \cdot \exp \left[ - \exp \left( \frac{\mu \cdot e}{A} (\lambda - t) + 1 \right) \right]$$

152 Where  $y(t)$  is a cumulative  $\text{CH}_4$  production (mL),  $A$  is the ultimate  $\text{CH}_4$  yield (mL),  $\mu$  is the  
153 maximum production of  $\text{CH}_4$  production rate (mL/day), and  $\lambda$  is the lag phase (day).

154 The methanogenic pathways during the substrates degradation (acetoclastic  
155 methanogenesis or hydrogenotrophic methanogenesis) were determined by the gas isotopic  
156 signature analysis. Periodically gas was sampled into a 7 mL vacuumed serum tubes for  
157 analysis of  $\delta^{13}\text{CH}_4$  and  $\delta^{13}\text{CO}_2$ . A Trace Gas Chromatograph Ultra (Thermo Scientific)  
158 attached to a Delta V Plus isotope ratio mass spectrometer via a GC combustion III (Thermo  
159 Scientific) was used to carry out the analysis. The principle of the method was described by  
160 (Brand, 1996; Sugimoto et al., 1991). The uncertainties, determined by replicate  
161 measurement, for  $\delta^{13}\text{CH}_4$  and  $\delta^{13}\text{CO}_2$  analysis was around 0.5 ‰. As indicator of the  
162 methanogenic pathway, the apparent isotopic factor ( $\alpha_{\text{app}}$ ) was calculated as presented in the  
163 following equation:

$$164 \quad \alpha_{\text{app}} = (\delta^{13}\text{CO}_2 + 10^3) / (\delta^{13}\text{CH}_4 + 10^3)$$

165 It is usually assumed that if the  $\alpha_{\text{app}}$  is superior to 1.065, the hydrogenotrophic way is  
166 the most important. On the contrary if the  $\alpha_{\text{app}}$  is inferior to 1.055, the methanogenesis is  
167 dominated by the acetoclastic way (Conrad, 2005; Whiticar et al., 1986).

## 168 2.5. Chemical analysis

169 Volatile Fatty Acids (VFA) concentrations were measured using ionic chromatography  
170 (ICS 5000+, Thermo Fisher Scientific) equipped with IonPAC ICE-AS1 column. The mobile  
171 phase was composed of heptafluorobutyric acid (0.4mmol/L) and tetrabutylammonium  
172 (5mmol/L). The VFA quantified were acetate, propionate, butyrate, valerate, formate, lactate  
173 and caproate.



174 Ammonium (NH<sub>4</sub><sup>+</sup>) concentration was measured using the Nessler's colorimetric  
175 method following the French standard (NF T 90-105) in spectroscopic tanks using Hach  
176 spectrometer DR2800. The link between Free Ammonia Nitrogen (FAN), Total Ammonia  
177 Nitrogen (TAN), pH and temperature can be summarized with the following equation  
178 (Anthonisen et al., 1976):

$$FAN = \frac{10^{pH}}{\left(\exp\left(\frac{6344}{T}\right) + 10^{pH}\right)} \times TAN$$

179 Where T is the temperature in Kelvin.

180 Dissolved organic and inorganic (DOC and DIC) carbons were measured following the  
181 French standard NF EN 1484 using a DOC analyser TOC-L Shimadzu.

182 Chemical oxygen demand (COD) was measured with LCK514 kit (Hach Lange)  
183 according to the manufacturer's instructions.

184 The carbon and nitrogen quantities contained in the substrates and inoculum were  
185 analysed on the crushed and dried sample. 10 mg of the sample was placed on sampler tin and  
186 analysed using an elementary analyser (VARIO EL III, Bioritech).

## 187 2.6. RNA extraction and 16S RNA sequencing analysis

188 Based on the methane production (Figure 2), a total of 19 samples were selected. Total RNA  
189 was extracted using the commercial kit FastRNA Pro™ Soil-Direct (MP Biomedicals)  
190 following the manufacturer's specifications. Then, DNA co-extracted was removed using  
191 TURBO™ DNase (Ambion) kit following the manufacturer's instructions. The RNA was  
192 denatured by 2 min at 85°C in a dry bath and was then stored on ice. RNA purification was  
193 carried out using the Agencourt AMPure RNA magnetic beads purification system (Beckman  
194 Coulter) by adding 1.8 volumes of beads by volume of RNA. After mixing by pipetting and 5  
195 min of incubation, beads were captured using a magnetic rack on one side of the tube and then  
196 washed by adding 500µL of 70% cold ethanol (diluted in DEPC-water). After incubation of

197 the tube during 30 seconds at room temperature, the ethanol was removed. This washing step  
198 was repeated 3 times. Once ethanol finally evaporated, beads were resuspended with DEPC-  
199 water to eluted RNA from the beads. Finally beads were removed using the magnetic rack and  
200 RNA was recovered in the supernatant. The integrity and quantity of the RNA was evaluated  
201 using the Hight Sensitivity RNA ScreenTape and 4200 TapeStation (Agilent Technologies)  
202 following the manufacturer's protocol.

203 A reverse transcription PCR (RT-PCR) was carried out on the RNA using the mix iScript  
204 Reverse Transcription Supermix (Biorad) and the following thermocycler program: 5min at  
205 25°C, 30min at 42°C and 5min at 85°C. The cDNA was quantified using Qubit 2.0  
206 fluorometer (ssDNA assay kit, Invitrogen, Life Technologies).

207 Archaeal hyper variable region V4-V5 of the 16S rRNA gene was amplified on the cDNA  
208 according to the protocol described by Madigou et al (Madigou et al., 2018).

209 16S rRNA tags reads were imported in FROGS. FROGS (Find Rapidly OTU with  
210 Galaxy Solution) is a galaxy/CLI workflow designed to produce an OTU count matrix from  
211 high depth sequencing amplicon data (Escudié et al., 2018). FROGS abundance file was  
212 examined using R CRAN software (version 3.4.4). Considering the dispersion in the total  
213 number of reads identified in each sample, archaeal OTUs abundances were normalized with  
214 total sum scaling. Only OTUs that exceeded 1% in at least one sample have been taken into  
215 account for the analysis.

216

### 217 3. Results and discussion

#### 218 3.1. Physico-chemical characteristics of the substrates and inoculum

219 The physico-chemical characteristics of the different substrates and the inoculum are  
220 summarised in Table 1. The C/N ratio of fish was lower than the C/N ratio of sludge and grass  
221 which were similar. Two key information can be drawn from these analyses. The first one is

222 the higher amount of nitrogen (N) in fish waste (9.5 %) compared to sludge and grass (2.46  
223 and 2.2 % respectively), explaining the low C/N ratio. This result implies a higher potential to  
224 produce ammonia during the fish degradation compared to sludge and grass. The second  
225 information is the lower quantity of dissolved organic carbon in sludge (1250 mgC/L)  
226 compared to fish (7921 mgC/L) and grass (7692 mgC/L). It suggests that even if the C/N ratio  
227 was similar between grass and sludge, the CH<sub>4</sub> production could start earlier in fish and grass  
228 because more readily available carbon was present.

229 **[[insert table 1]]**

230 A BMP test was carried out to determine the maximal CH<sub>4</sub> production potential for the  
231 different substrates. The kinetics production parameters determined after modelling of the  
232 data with Gompertz equation are presented in the Table 2. Degradation started immediately  
233 for all the substrates as expected according to the substrate/inoculum ratio used. Sludge-BMP  
234 degradation was the slowest (2.09 mgC/D/gCOD) and the less important (44.35 mgC/gCOD  
235 of CH<sub>4</sub> was produced). The slow degradation of the sludge was in accordance with the lower  
236 DOC initial value observed for the sludge. Fish-BMP and Grass-BMP degradation produced  
237 almost the same final amount of CH<sub>4</sub> (circa 119 mgC/gCOD) and at a similar rate (circa 8  
238 mgC/D/gCOD).

239 **[[insert table 2]]**

### 240 3.2. Mono-digestion of the substrates

241 The specific methane production of wastewater sludge (S-100), fish waste (F-100) and  
242 grass (G-100) mono-digestion are presented in the Fig. 1 and the kinetics parameters of  
243 production determined after modelling with Gompertz equation are presented in the Table 2.  
244 Between 30 to 50 % of the initial quantity was transformed into biogas. Wastewater sludge in  
245 the experimental batch mono-digestion produced the lowest final CH<sub>4</sub> quantity (46.62  
246 mgC/gCOD) despite a similar COD amount fed to the bioreactor at the beginning of the

247 experiment. The highest final CH<sub>4</sub> production was observed for fish (F-100, 87.55  
248 mgC/gCOD, compared to respectively 68.43 and 46.62 mgC/gCOD for G-100 and S-100)  
249 while the production began earlier and was faster when grass was used as a single substrate  
250 (G-100). The faster CH<sub>4</sub> production rate estimated by Gompertz modelling for G-100 can be  
251 explained by the higher amount of DOC present in the grass (supplementary Figure B.1-A).  
252 Because dissolved organic carbon was readily available, the CH<sub>4</sub> production could start earlier  
253 and be faster. It is interesting to notice that the kinetic of CH<sub>4</sub> production differed between  
254 fish and grass during mono-digestion experiment while they were similar during the BMP  
255 test. Because the only difference between the mono-digestion experiment and the BMP test  
256 was the Substrate/Inoculum ratio, respectively 12/1.2 and 0.7/7 gCOD, it can be hypothesised  
257 that the concentration of the methanogenic biomass at the beginning of the experiments  
258 played a non-negligible role in the kinetics of the CH<sub>4</sub> production. This assessment was  
259 already described in several publications (Hobbs et al., 2018; Zhou et al., 2011).

260 Biogas productions observed in our experiment was compared to the values described in  
261 the literature for similar substrates. Abendroth et al observed a higher performance of  
262 methane production from WAS mono-digestion (250-300 mL/gCOD) (Abendroth et al.,  
263 2017) than in our study (95 mL/gCOD). This difference can be explained by the difference of  
264 WAS quality according to the preprocess. Indeed it was already described that industrial  
265 digesters treating WAS present different performances (Rivière et al., 2009; Sundberg et al.,  
266 2013). In the same way grass anaerobic digestion performances will greatly depends of its  
267 type, treatment or freshness (Prochnow et al., 2009). Fish mono-digestion methane  
268 performances will also depends of the type and the part of fish digested (Donoso-Bravo et al.,  
269 2015). However in our study the final methane production (198 mL/gCOD) was comparable  
270 to the methane performances obtained by Donoso-Bravo et al which was around 200-300  
271 mL/gCOD.

272 **[[insert figure 1]]**

273 The concentration of acetic and propionic acids for all batches are presented in the  
274 supplementary figure B.1-B. The acetate maximum accumulation during sludge mono-  
275 digestion S-100 was the lowest (47 mgC/L/gCOD) and acetate took around 40 days to be  
276 degraded. In comparison, fish and grass mono-digestion (F-100 and G-100) degradation  
277 produced a similar level of acetate in 7 days (circa 90 mgC/L/gCOD) suggesting that organic  
278 carbon in fish and grass was more readily degradable. However the total acetate degradation  
279 occurred in 13 days for G-100 and in 43 days for F-100. These results coupled to the  
280 difference in the final methane production between G-100 and F-100 indicated a lower  
281 degradability across time of the grass compared to the fish. No butyrate was produced during  
282 the sludge degradation while butyrate accumulation was mainly observed in F-100 (maximum  
283 30 mgC/L/gCOD compared to 10 mgC/L/gCOD in G-100). The propionate maximum  
284 accumulation was more important for F-100 (47 mgC/L/gCOD) compared to G-100 (37  
285 mgC/L/gCOD). For G-100, the degradation of the propionate started after all the acetate had  
286 been totally degraded. In F-100 the degradation was not completed at day 72. An increase of  
287 15 mgC/gCOD of CH<sub>4</sub> was observed between day 70 and the end of the experiment  
288 suggesting that a part of the 25 mgC/gCOD of propionate present at day 70, started to be  
289 degraded after day 70. Propionate is one of the most important precursors in methane  
290 production after acetate (Lawrence and McCarty, 1969) but it is also reported to accumulate  
291 easily and cause process inhibition in some cases (Gallert and Winter, 2008; Wang et al.,  
292 2009). Anaerobic oxidation of propionic acid is thermodynamically unfavorable and depends  
293 on acetate and H<sub>2</sub> content (Boone and Bryant, 1980; Mawson et al., 1991). It is only  
294 performed by specific microorganisms. The major pathway for the anaerobic propionate  
295 degradation is a syntrophic degradation of propionate linked to H<sub>2</sub> transfer via a methanogen  
296 (Ariesyady et al., 2007). Delays observed in the degradation of the propionate for fish mono-

297 digestion F-100 could be explained by the time needed by the appropriate microorganism to  
298 grow and by the syntrophy to take place.

299 The evolution of  $\text{NH}_4^+$ , pH and  $\text{NH}_3$  values during the substrates degradation are  
300 presented in the supplementary figure B.1-C. F-100 produced in 7 days around 1300 mg/L of  
301  $\text{NH}_4^+$  compared with 350 mg/L for G-100 and S-100 corresponding to a respectively amount  
302 of 140 and 30 mg/L of  $\text{NH}_3$ . Ammonia nitrogen is known to be an inhibitor of the AD,  
303 especially free ammonia ( $\text{NH}_3$ ) (Fotidis et al., 2013; Rajagopal et al., 2013). However a wide  
304 range of half inhibitory concentrations has been reported between 1.7 to 19 g/L of  $\text{NH}_4^+$   
305 (Chen et al., 2008; Poirier et al., 2016) and 50-1400 mg $\text{NH}_3$ /L (Rajagopal et al., 2013)  
306 depending on multiple factors such as the microbial community, temperature... The highest  
307 free ammonia accumulation observed for F-100 was 140 mg/L at day 50. The amount of  
308 ammonia observed in F-100 was under the inhibitory values described in the literature. It  
309 cannot be excluded that the microbial community was partly inhibited, particularly the  
310 methanogens and the propionate degrading acetogenic bacteria which are known to be  
311 sensitive to free ammonia (Calli et al., 2005; Westerholm et al., 2011).

312

### 313 3.3. Co-digestion of the substrates

#### 314 3.3.1 Performances of wastewater sludge co-digestion with fish waste

315 Evolution of the cumulated  $\text{CH}_4$  production over time for the different mixtures is  
316 presented in Figure 2 and the Table 2 details the results of the Gompertz modelling for each  
317 mixture. In all cases the addition of fish enabled to increase the final  $\text{CH}_4$  production  
318 compared to S-100. For example, F-25, which contains 25 % of fish and 75 % of sludge,  
319 produced 65.58 mgC/gCOD of  $\text{CH}_4$ , *ie.* 18 mgC/gCOD more than S-100. According to the  
320 Gompertz modelling the latency before  $\text{CH}_4$  production start was not significantly modified  
321 between the different mixtures, but the production rate was increased from 2.31 to 3.00

322 mgC/D/gCOD for F-25 and F-75 respectively. Dissolved organic carbon accumulation  
323 (supplementary figure C.1-A) between days 0 to 7, representative of the solid carbon  
324 degradation during the early hydrolytic phase, increased when more than 25 % (gCOD) of  
325 fish was mixed to WAS. This could be explained by the presence of a higher quantity of  
326 easily degradable carbon or by a hydrolysis step faster when fish was present than for S-100.

327 Volatile fatty acids accumulation, presented in the supplementary figure C.1-B show a  
328 similar VFA pattern evolution for the mixtures F-25 and F-50 than for S-100. The acetate  
329 accumulation profile in F-75 was also similar to S-100 while the propionate maximum  
330 accumulation was closed to F-100 (45 mgC/L/gCOD). The consumption of the propionate in  
331 F-75 was completely achieved after day 60 while in F-100 the propionate degradation had not  
332 started yet. Regarding the butyrate production no significant effect of AcoD was observed.  
333 The ammonia accumulation presented in the supplementary figure C.1-C shows an increase of  
334 the  $\text{NH}_4^+$  level proportional to the amount of fish added in the feeding, indicating that  
335 ammonia production was mainly due to fish. Use wastewater sludge to dilute fish waste  
336 decreased the  $\text{NH}_4^+$  level which seemed to reduce the inhibition of the propionate degrading  
337 population observed on the fish mono-digestion. This allowed to increase the propionate  
338 degradation rate and to produce the  $\text{CH}_4$  faster.

### 339 3.3.2 Performances of wastewater sludge co-digestion with garden-grass

340 In the case of co-digestion with grass, the final  $\text{CH}_4$  production was increased only  
341 when more than 50 % of grass was mixed with sludge compared to sludge mono-digestion  
342 (Fig. 2). G-25 presented an earlier  $\text{CH}_4$  production but a final production similar to S-100.  
343 The more the proportion of grass was important the more the latency to produce  $\text{CH}_4$  was  
344 reduced (from 4.5 days to 2.6 for G-25 and G-75 respectively). Mixing grass and sludge  
345 increased the amount of dissolved organic carbon accumulated during the 7 first days. All the  
346 mixtures presented the same evolution of acetate accumulation than G-100 with a higher and

347 faster accumulation than in S-100. However, propionate final accumulation was 2 times  
348 higher for the mixtures than for S-100 (20 mgC/L/gCOD) and G-100 (37 mgC/L/gCOD) but  
349 propionate degradation for all bioreactors was achieved in 30 days. For all bioreactors of grass  
350 co-digestion, the ammonia level stayed stable along the experiment closed to 350mg/L such  
351 as the level in S-100 and G-100.

352 **[[insert figure 2]]**

353

#### 354 3.4. Influence of the co-digestion on the methanogenic pathways

355 Measurement of the biogas carbon stable isotopic composition allowed to follow the  
356 evolution of the methanogenic pathway across time (Conrad, 2005). The results are presented  
357 in the figure 3.

358 The methane production in S-100 was carried out during the first 20 days mostly by the  
359 hydrogenotrophic pathway ( $\alpha_{app}$  superior to 1.065). After 20 days, the methanogenic pathway  
360 changed progressively from hydrogenotrophic to acetoclastic pathway ( $\alpha_{app}$  inferior than  
361 1.055 after 30 days).

362 In the fish mono-digestion the gas production at the beginning of the experiment was  
363 dominated by the acetoclastic pathway. During the first week of the experiment, the  
364 methanogenic pathway increased from 1.04 to 1.06, namely from acetoclastic pathway to a  
365 mix of methanogenic pathways. This can be explained by a high hydrolytic activity producing  
366 H<sub>2</sub> and CO<sub>2</sub> and favouring the gas production by the hydrogenotrophic pathway. During the  
367 next 20 days the  $\alpha_{app}$  decreased due to the consumption of the acetate by the acetoclastic  
368 methanogens. Finally, when all the acetate was degraded, the  $\alpha_{app}$  increased again due to the  
369 syntrophic oxidation of the propionate during which methane was produced by acetoclastic  
370 and hydrogenotrophic pathways. The evolution across time of the methanogenic pathways for  
371 the mixtures of the co-digestion with fish followed the same evolution than F-100 but with the



372 values of the  $\alpha_{\text{app}}$  corresponding to an intermediate between F-100 and S-100. Because the  
373 propionate was not degraded at the same time for the different fish mixtures, the  $\alpha_{\text{app}}$  evolved  
374 differently between the different mixtures. This study shows that addition of fish waste  
375 influenced strongly the methanogenic pathway during the co-digestion.

376 The mono-digestion of grass presented a similar evolution across time of the  
377 methanogenic pathway. The beginning of the experiment started in the acetoclastic pathway.  
378 After a first increase followed by a decrease of the  $\alpha_{\text{app}}$  induced by the production of  $\text{CO}_2$  then  
379 the consumption of the acetate, the  $\alpha_{\text{app}}$  stabilised at 1.055 due to the propionate degradation.  
380 Contrary to the fish mixtures, the co-digestion mixtures of grass presented a distinction  
381 between two groups. The methanogenic pathway evolution for G-25 was closer to S-100 with  
382 a beginning in the hydrogenotrophic pathway while the methanogenic pathways evolution of  
383 G-50 and G-75 was similar to G-100. This differentiation in the methanogenic pathways for  
384 the grass mixtures can explain the difference observed in the methane production.

385 **[[insert figure 3]]**

386 In support of the isotopic fractionation results the active archaeal community was  
387 analysed using 16S RNA sequencing during the methane production phase. *Methanosarcina*  
388 genus was the most abundant archaea in all the digesters independently of the feeding  
389 composition. This archaea has a versatile methanogenesis metabolism but in regards with the  
390 isotopic results the acetoclastic pathway seemed to be dominant. Indeed, except for sludge  
391 mono-digestion, the methane was mostly produced by the acetoclastic pathway since the  
392 beginning of the experiments. Other hydrogenotrophic archaea were found in the digesters.  
393 *Methanoculleus* and *Methanobacterium* genera were found in digesters fed with sludge and/or  
394 fish. Their abundances were higher in sludge mono-digestion at the beginning of the methane  
395 production (day 14) than in fish fed digesters. This result is in accordance with the isotopic  
396 fractionation which showed that hydrogenotrophic pathway was dominant in sludge mono-

397 digestion during the 20<sup>th</sup> first days. *Methanospirillum* were relatively abundant in digesters fed  
398 with grass and sludge, reaching up to 25% in G25 bioreactors. Archaea of *Methanofollis* and  
399 *Methanocorpusculum* were found specifically in digesters fed with more than 25 of grass but they  
400 remained minority.

401 **[[insert figure 4]]**

402

### 403 3.5. Estimation of the optimal blend of the co-digestion using indicators

404 In order to evaluate the benefit of the co-digestion, the experimental values of the CH<sub>4</sub>  
405 production measured for the bioreactors were compared to two indicators that we built. The  
406 first one is the empirical maximum production that could be obtained under optimal  
407 conditions (determined with BMP) and called thereafter Optimal Digestion Indicator (ODI).  
408 For the different mix, ODI was calculated as a linear combination of the BMP experimental  
409 values obtained for Sludge-BMP, Fish-BMP and Grass-BMP using the percentage of each co-  
410 substrate as coefficient. The second indicator is the empirical production that could be  
411 obtained under experimental conditions and called Mono-Digestion Indicator (MDI). MDI  
412 was calculated in the same way than ODI but using the linear combination of the mono-  
413 digestion experimental values. Figure 5 represents the comparison of the experimental CH<sub>4</sub>  
414 production profiles to the indicators for each mixture. The Table 2 summarises the kinetics  
415 production parameters determined after modelling the data with Gompertz equation for all the  
416 experiments and indicators.

417 The comparison of the experimental mono-digestion (F-100, G-100 and S-100) to the  
418 Optimal Digestion Indicator was already described in details in the section dedicated to the  
419 mono-digestion. The ODI was higher than the experimental methane production but the co-  
420 digestion with fish or grass allowed to get close to the final methane production and/or the  
421 production rate of the mixtures to the ODI.

422 For the co-digestion with fish waste the comparison showed no significant differences  
423 between the experimental final methane production and the MDI. However the methane  
424 production rate for the F-75 (3.00 mgC/days/gCOD) was increased comparing to the MDI  
425 (2.33 mgC/days/gCOD). This result, taken with the chemical results (propionate) and the  
426 methanogenic pathway analysis, indicates that use of 75 % of fish seemed to be the optimal  
427 proportion to enhance the methane production from sewage sludge. Indeed, the propionate  
428 was consumed earlier and was associated to a faster return to the acetoclastic methanogenic  
429 pathway. On an industrial point of view if the aim is to enhance the final CH<sub>4</sub> production from  
430 wastewater sludge, fish waste is a good candidate even at a low quantity. The other advantage  
431 to treat fish waste in co-digestion is to limit the risk of an inhibition by the ammonia and the  
432 propionate which are accumulated during the degradation in mono-digestion. The high  
433 potential of VFA production from fish was already studied (Bermúdez-Penabad et al., 2017).  
434 Even if no such inhibition was observed in our system, the potentiality of ammonia and VFA  
435 accumulation was observed. However, in case of CH<sub>4</sub> production enhancement, the VFA  
436 production can inhibit the system and the co-digestion can be a solution to overcome this  
437 inhibition (Xu et al., 2017).

438 Regarding the co-digestion with grass, G-25 maximal methane production was slightly  
439 lower than the one estimated by the MDI. In contrast, G-50 and G-75 allowed to increase the  
440 maximal production by 1.2 times and the CH<sub>4</sub> production rate from 1.2 and 1.3 respectively.  
441 Because the experimental CH<sub>4</sub> production was higher than the MDI prediction during all the  
442 experiment for G-50 and G-75, it can be supposed that a synergistic effect occurred between  
443 grass and wastewater sludge at these proportions. The mixture improving the AD  
444 performances the more in term of CH<sub>4</sub> production is G-75. The substrate degradation started  
445 earlier, the methane production rate was improved by 1.2 times and the maximal production

446 was increased by 1.7 times compared to the mono-digestion of wastewater of sludge and 0.9  
447 compared to the mono-digestion of grass.

448 **[[insert figure 5]]**

449

#### 450 4. Conclusion

451 Increasing the fish concentration in co-digestion with wastewater sludge gradually  
452 improved the final methane production up to 1.9 times when 75 % of fish was added. On the  
453 contrary grass enabled to improve the final methane production from WAS only when more  
454 than 25 % of grass was added. Adding more than 50 % of grass improved both the production  
455 rate and the final production by to 1.5 and 1.7 times respectively. Specifically built indicators  
456 showed that using 75 % of fish or grass as co-substrate with sewage sludge enabled to obtain  
457 the maximum final methane production. In nearly all the bioreactors, archaea from  
458 *Methanosarcina* genus accounted for more than 75% of the archaeal diversity. No significant  
459 difference in the methanogenic pathways was observed across time between fish and grass  
460 mono-digestion. It was mostly acetoclastic while wastewater sludge mono-digestion changed  
461 from hydrogenotrophic to acetoclastic methanogenesis pathway. The anaerobic co-digestion  
462 allowed to limit the variation between the methanogenesis pathway of the sludge.

463

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473

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614

615 **Table 1. Characteristics of substrates and inoculum**

	<b>Wastewater Sludge</b>	<b>Fish</b>	<b>Grass</b>	<b>Inoculum</b>
NH <sub>4</sub> <sup>+</sup> (mgN/L)	299	899	438	628
DOC (mgC/L)	1250	7921	7692	149
DIC(mgC/L)	99	346	424	753
COD (gO <sub>2</sub> /L)	103	310	95	13
C (%)	41.58	43.67	42.55	22.58
N (%)	2.46	9.50	2.20	2.19
C/ N	16.89	4.60	19.37	10.29
Dry matter (DM) (%)	5	24	11	1
Volatile matter (VM) (%)	81	79	84	61
Lactate (mgC/L)	0.00	0.00	398.80	23.40
Formate (mgC/L)	0.00	138.52	0.00	0.00
Acetate (mgC/L)	537.08	62.88	11.08	2.08
Propionate (mgC/L)	441.83	0.00	0.00	0.00
Butyrate (mgC/L)	199.20	0.00	0.00	0.00
Valerate (mgC/L)	43.88	0.00	0.00	0.00

616

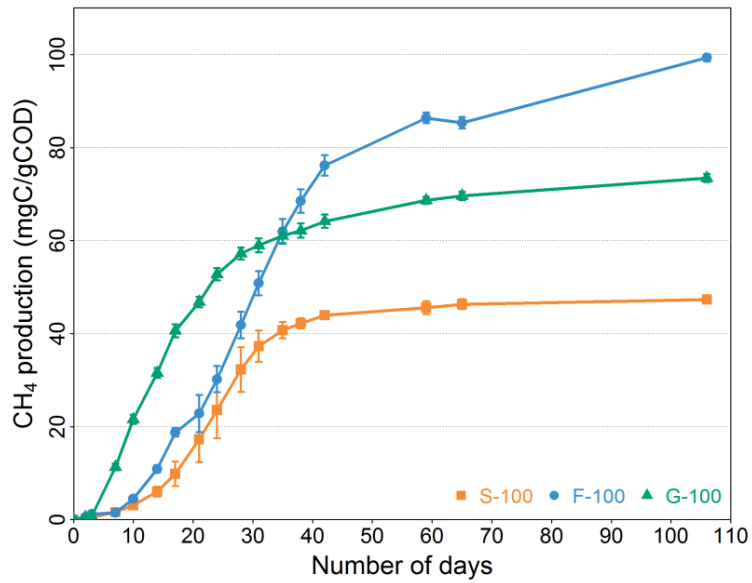
617

618 **Table 2. Kinetics parameters for CH<sub>4</sub> production using Gompertz model for the**  
619 **different mixtures of substrates, BMP tests and the biodegradation indicators.** The data  
620 are the mean values for the triplicate bioreactors, standard deviations are indicated.  $\mu$   
621 correspond to the CH<sub>4</sub> production rate,  $\lambda$  to the latency and A to the maximum production.

	<b>Name</b>	<b><math>\mu</math> (mgC/day/gCOD)</b>	<b><math>\lambda</math> (day)</b>	<b>A (mgC/gCOD)</b>
<b>Experimental Batches</b>	F-100	2.35 ( $\pm$ 0.57)	10.1 ( $\pm$ 0.8)	87.55 ( $\pm$ 13.00)
	F-75	3.00 ( $\pm$ 0.11)	10.8 ( $\pm$ 0.6)	86.31 ( $\pm$ 4.93)
	F-50	2.49 ( $\pm$ 0.35)	7.7 ( $\pm$ 0.8)	72.03 ( $\pm$ 4.49)
	F-25	2.31 ( $\pm$ 0.10)	9.4 ( $\pm$ 0.5)	64.65 ( $\pm$ 1.46)
	S-100	2.26 ( $\pm$ 0.26)	12.8 ( $\pm$ 1.3)	46.62 ( $\pm$ 0.8)
	G-25	1.93 ( $\pm$ 0.10)	4.5 ( $\pm$ 0.6)	42.75 ( $\pm$ 5.41)
	G-50	2.97 ( $\pm$ 0.21)	3.2 ( $\pm$ 1.1)	70.80 ( $\pm$ 4.72)
	G-75	3.45 ( $\pm$ 0.46)	2.6 ( $\pm$ 0.7)	79.30 ( $\pm$ 6.99)
	G-100	2.81 ( $\pm$ 0.12)	3.1 ( $\pm$ 0.2)	68.43 ( $\pm$ 0.97)
	<b>Mono-Digestion Indicator</b>	F75-MDI	2.33 ( $\pm$ 0.49)	10.8 ( $\pm$ 0.9)
F50-MDI		2.31 ( $\pm$ 0.43)	11.5 ( $\pm$ 1.1)	67.09 ( $\pm$ 6.90)
F25-MDI		2.28 ( $\pm$ 0.35)	12.1 ( $\pm$ 0.5)	56.85 ( $\pm$ 3.85)
G25-MDI		2.40 ( $\pm$ 0.24)	10.4 ( $\pm$ 1.0)	52.07 ( $\pm$ 0.84)
G50-MDI		2.54 ( $\pm$ 0.20)	8.0 ( $\pm$ 0.8)	57.53 ( $\pm$ 0.89)
G75-MDI		2.67 ( $\pm$ 0.16)	5.5 ( $\pm$ 0.5)	62.98 ( $\pm$ 0.93)
<b>BMP test</b>	Fish-BMP	8.18 ( $\pm$ 0.24)	0	119.35 ( $\pm$ 15.28)
	Sludge-BMP	2.09 ( $\pm$ 0.17)	0	44.35 ( $\pm$ 18.3)
	Grass-BMP	8.47 ( $\pm$ 3.67)	0	118.33 ( $\pm$ 12.9)
<b>Optimal Digestion Indicator</b>	F75-ODI	6.66 ( $\pm$ 0.22)	0	100.60 ( $\pm$ 16.04)
	F50-ODI	5.14 ( $\pm$ 0.21)	0	81.85 ( $\pm$ 16.79)
	F25-ODI	3.61 ( $\pm$ 0.19)	0	63.10 ( $\pm$ 17.55)
	G25-ODI	6.88 ( $\pm$ 2.80)	0	99.84 ( $\pm$ 14.25)
	G50-ODI	5.28 ( $\pm$ 1.92)	0	81.34 ( $\pm$ 15.60)
	G75-ODI	3.69 ( $\pm$ 1.05)	0	62.85 ( $\pm$ 16.95)

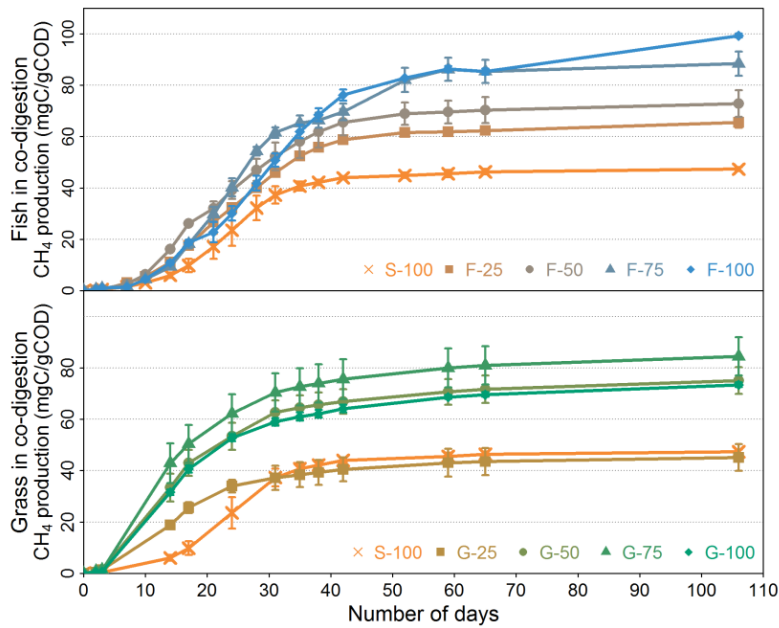
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625 **Figure 1. Cumulated CH<sub>4</sub> production (mgC/gCOD) over time (days) for the different**  
 626 **substrates in mono-digestion experiments.** Mean values of the triplicate bioreactors for CH<sub>4</sub>  
 627 productions, error bars represent standard deviation within triplicates. S, F and G stand for  
 628 Sludge, Fish and Grass respectively.



630

631 **Figure 2. Cumulated CH<sub>4</sub> production (mgC/gCOD) over time (days) for Fish and Grass**632 **used as co-substrates in co-digestion with wastewater sludge.** Mean values of the triplicate

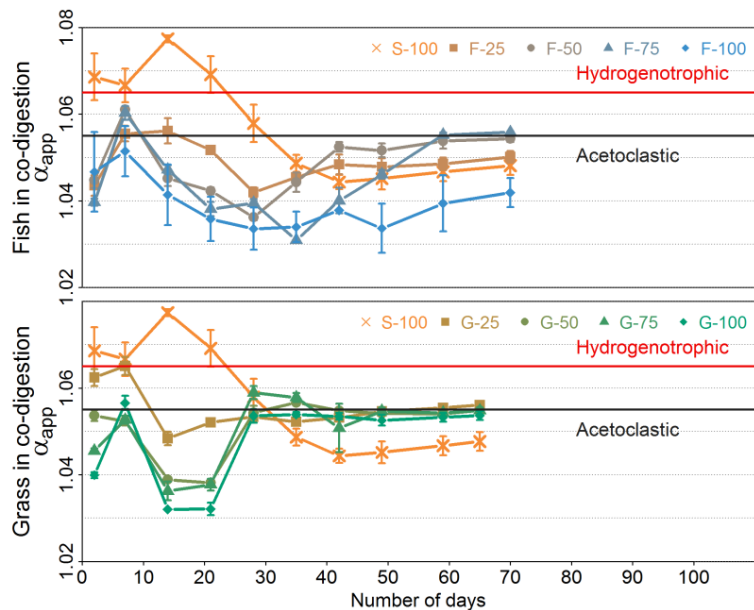
633 bioreactors, error bars represent standard deviation within triplicates. S-100 stands for

634 wastewater sludge alone, F-25, F-50, F-75, F-100 stands for respectively 25, 50, 75 or 100%

635 of fish (F) in co-digestion with sludge, G-25, G-50, G-75, G-100 stands for respectively 25,

636 50, 75 or 100% of Grass (G) in co-digestion with sludge.

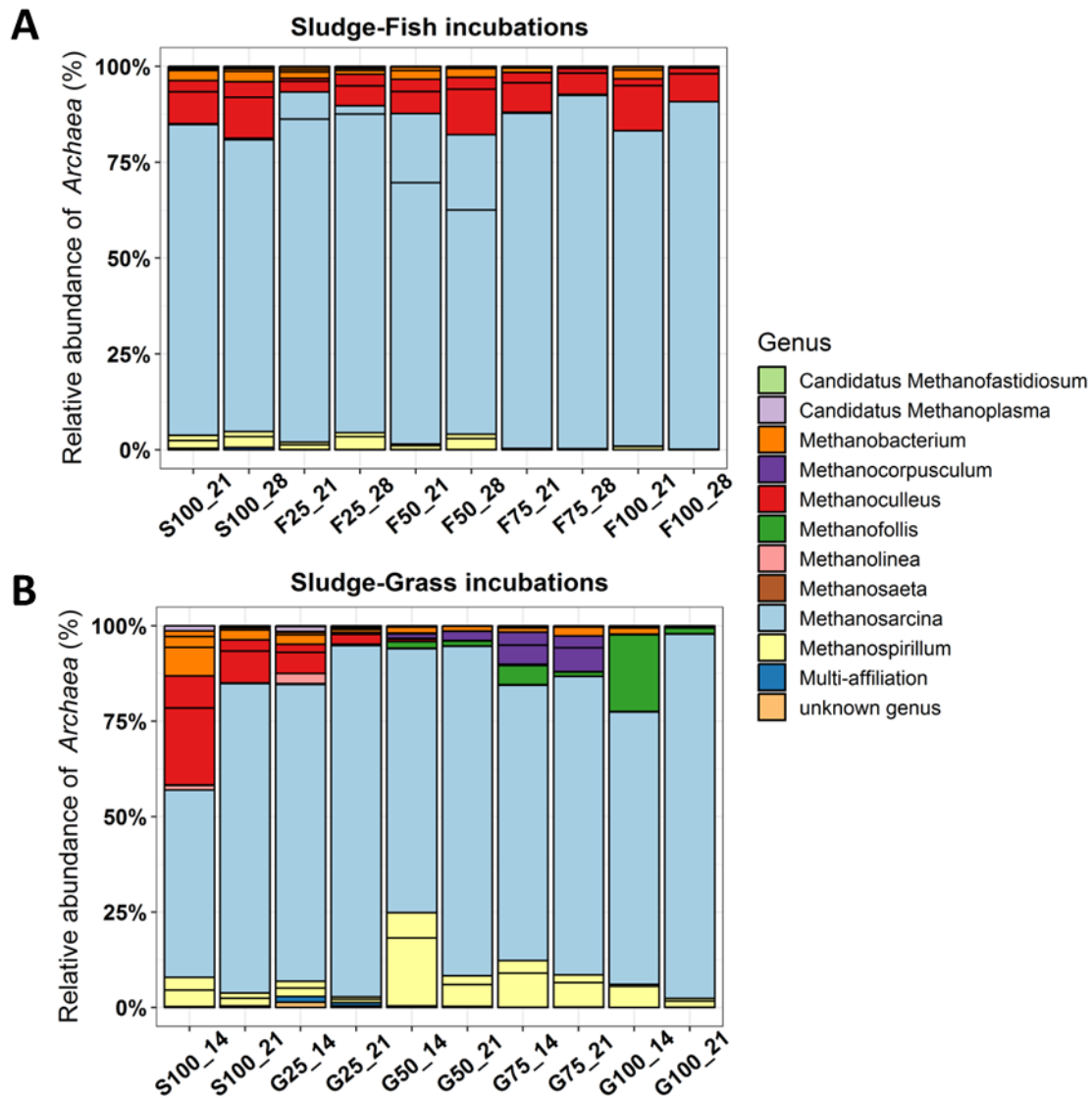
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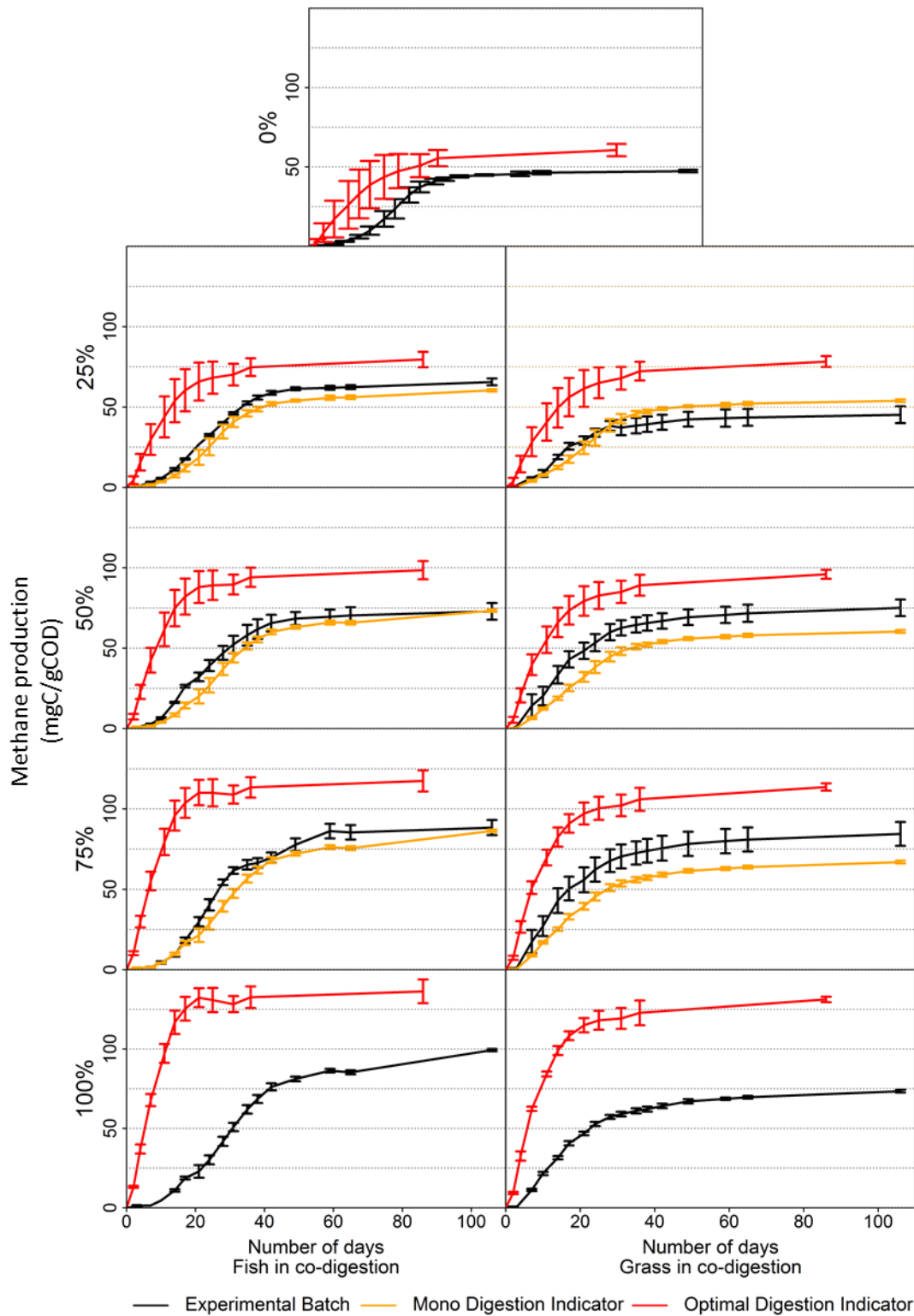
641 **Figure 3. Apparent isotope fractionation over time (days) for Fish and Grass used as co-**  
642 **substrates in co-digestion with wastewater sludge.** Mean values of the triplicate  
643 bioreactors, error bars represent standard deviation within triplicates. S-100 stands for  
644 wastewater sludge alone, F-25, F-50, F-75, F-100 stands for respectively 25, 50, 75 or 100%  
645 of fish (F) in co-digestion with sludge, G-25, G-50, G-75, G-100 stands for respectively 25,  
646 50, 75 or 100% of Grass (G) in co-digestion with sludge.

647



648  
 649 Figure 4: Taxonomic composition at genus level based on the 16S archaea-specific amplicon  
 650 sequences. (A) Samples from reactors fed with sludge and/or fish at days 21 and 28 of the  
 651 experiment (B) Samples from reactors fed with sludge and/or grass at days 14 and 21 of the  
 652 experiment. Days were selected to correspond to the methane production phase. S100 stands  
 653 for wastewater sludge alone, F25, F50, F75, F100 stands for respectively 25, 50, 75 or 100%  
 654 of fish (F) in co-digestion with sludge, G25, G50, G75, G100 stands for respectively 25, 50,  
 655 75 or 100% of Grass (G) in co-digestion with sludge.





656

657 **Figure 5. Experimental Batch methane production (mgC/gCOD) compared to the**  
 658 **Maximal Digestion Indicator and the Optimal Digestion Indicator over time (days) for**  
 659 **fish and grass substrates in co-digestion with wastewater sludge. Mean values of the**

660 triplicate bioreactors for CH<sub>4</sub> productions, error bars represent standard deviation within  
661 triplicates. S-100 stands for wastewater sludge alone, F-25, F-50, F-75, F-100 stands for  
662 respectively 25, 50, 75 or 100% of fish (F) in co-digestion with sludge, G-25, G-50, G-75, G-  
663 100 stands for respectively 25, 50, 75 or 100% of Grass (G) in co-digestion with sludge.

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