

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

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# ► To cite this version:

Laëtitia Cardona, Camille Levrard, Angéline Guenne, Olivier Chapleur, Laurent Mazéas. Co-digestion of wastewater sludge: choosing the optimal blend. Waste Management, 2019, 87, pp.772-781. 10.1016/j.wasman.2019.03.016 . hal-02547814

# HAL Id: hal-02547814 https://hal.inrae.fr/hal-02547814

Submitted on 20 Apr 2020  $\,$ 

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- 19 Declarations of interest: none
- 20

## 21 Abstract

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

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

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# 41 Keywords

Anaerobic digestion - methanogenic pathways – grass – fish – carbon-isotopic fractionation
 43

44 1. Introduction

Anaerobic Digestion (AD) is a multistep biological process allowing to convert various types of organic waste into a renewable energy, the biogas (composed of  $CH_4$  and  $CO_2$ ) and digestate. This bioprocess has been used for more than a century, in particular to stabilise the wastewater sludge (WAS) obtained from wastewater treatment plants (Astals et al., 2012). It is particularly attractive as it allows to simultaneously produce energy in the form of biogas and to reduce the volume of sludge (Luostarinen et al., 2009). However, the benefit of using only WAS to produce methane by AD is limited by its low C/N ratio and low digestion
efficiency, leading to low CH<sub>4</sub> production yield (Astals et al., 2013; Park et al., 2016).

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

Different waste have already been successfully tested to improve WAS anaerobic digestion. In association with slaughterhouse waste, Borowski & Kubacki (2015) succeeded in increasing the specific methane production by 2-fold when 50 % of slaughterhouse waste was mixed to WAS at an organic loading rate (OLR) of 4 kgVS/m<sup>3</sup>d. Neither ammonia nor LCFA inhibition was observed and an effect of VFA accumulation was observed only when the OLR was superior to 4 kgVS/m<sup>3</sup>d. Wickham et al. (2016) tested several waste such as food waste, paper pulp, fat-grease-oil (FOG) waste and dehydrated *Ulva* macroalgae. Each substrate was mixed at different ratio with WAS (5, 10 and 15 % by weight). Final methane
production was increased by three to six times thanks to the co-digestion compared to monodigestion of WAS.

79 In this study, different mixtures of wastewater sludge (WAS) with garden-grass (GG) or fish waste (FW) as co-substrates were tested to determine the optimal blend allowing for the 80 most efficient  $CH_4$  production. Total fish production in the world has expanded since the last 81 five decades from 20 million tons in 1960 to 167.2 million tons in 2014 mainly due to the 82 increase of the aquaculture production (FAO, 2016). The amount of waste provided by the 83 fishery industries (as canneries) is important. Some authors evaluated the possibility to use 84 different parts of the fish (skins, viscera, bones ...) (Donoso-Bravo et al., 2015), or different 85 fish species (Eiroa et al., 2012) for methane production with high biodegradability level. 86 However, this type of waste can be rich in protein and/or lipids inducing ammonia and/or 87 88 LCFA inhibition as observed by Eiroa et al. (2012). Literature on the possibility to use fish waste as co-substrate in AD is still scarce and to the best of our knowledge co-digestion of 89 fish waste with wastewater sludge was not studied yet. 90

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

99 The aim of this study is to investigate the possibility for improving wastewater sludge100 degradation during anaerobic co-digestion with fish waste or garden grass. Degradation

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

106

107 2. Methods

108 2.1. Feedstock preparation and characterisation

Wastewater sludge came from an industrial wastewater treatment plant (Valenton,
France). Two organic co-substrates were tested. Fish waste was collected from a fish shop and
grass from the mowing of the Institute's lawn. Both waste were crushed and the solid part was
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.

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

119 2.2. Co-digestion experimental set-up

In total 27 anaerobic batch bioreactors were set-up using 1 L glass bottles (700 mL working volume). Each digester was inoculated with methanogenic sludge and fed with a mixture of a main substrate (wastewater sludge) and one co-substrate (fish waste or grass) to reach a substrate/inoculum ratio of 12 gCOD/ 1.2 gCOD. Different ratios of main substrate /co-substrate were tested (25/75, 50/50, 75/25) as detailed in the supplementary Table A.1. Controls with 100 % of wastewater sludge, fish waste or grass were also carried out. All the digesters were complemented with a biochemical potential buffer (International Standard ISO 11734 (1995)) to reach a final working volume of 700 mL. All incubations were performed in triplicate. The bioreactors were then sealed with a screw cap and a rubber septum. The headspaces were flushed with N<sub>2</sub> (purity > 99.99 %, Linde gas SA) and the bottles were incubated at 35°C in the dark and without agitation.

Weekly, 6 mL of liquid phase were sampled through the septum using a syringe and centrifuged at 10,000 g for 10 minutes. The supernatant and he pellet were snap frozen and 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 maximal production under optimal parameters. The ratio substrate/inoculum used in BMP test 136 was 0.7 gCOD/7 gCOD to limit the latency due to the microbial growth. The biochemical 137 138 potential buffer previously mentioned was used to reach a final volume of 500 mL in 1L glass bottles. As for the batch experiment, bottles were sealed, flushed with  $N_2$  and incubated at 139 140 35°C in the dark without agitation. The experiment was made in triplicate. Gas production and composition were followed over time. A control containing only the inoculum was 141 carried out in parallel and the biogas production of this control was taken into account to 142 calculate the substrates gas productions. The mixtures details are presented in the 143 supplementary Table A.1. 144

145 2.4. Gas production and stable carbon isotope signature

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

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

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

Were y (t) is a cumulative CH<sub>4</sub> production (mL), A is the ultimate CH<sub>4</sub> yield (mL),  $\mu$  is the maximum production of CH<sub>4</sub> production rate (mL/day), and  $\lambda$  is the lag phase (day).

The methanogenic pathways during the substrates degradation (acetoclastic 154 methanogenesis or hydrogenotrophic methanogenesis) were determined by the gas isotopic 155 signature analysis. Periodically gas was sampled into a 7 mL vacuumed serum tubes for 156 analysis of  $\delta^{13}$ CH<sub>4</sub> and  $\delta^{13}$ CO<sub>2</sub>. A Trace Gas Chromatograph Ultra (Thermo Scientific) 157 attached to a Delta V Plus isotope ratio mass spectrometer via a GC combustion III (Thermo 158 Scientific) was used to carry out the analysis. The principle of the method was described by 159 (Brand, 1996; Sugimoto et al., 1991). The uncertainties, determined by replicate 160 measurement, for  $\delta^{13}CH_4$  and  $\delta^{13}CO_2$  analysis was around 0.5 ‰. As indicator of the 161 methanogenic pathway, the apparent isotopic factor  $(\alpha_{app})$  was calculated as presented in the 162 following equation: 163

164 
$$\alpha_{app} = (\delta^{13}CO_2 + 10^3) / (\delta^{13}CH_4 + 10^3)$$

165 It is usually assumed that if the  $\alpha_{app}$  is superior to 1.065, the hydrogenotrophic way is 166 the most important. On the contrary if the  $\alpha_{app}$  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

Volatile Fatty Acids (VFA) concentrations were measured using ionic chromatography (ICS 5000+, Thermo Fisher Scientific) equipped with IonPAC ICE-AS1 column. The mobile phase was composed of heptafluorobutyric acid (0.4mmol/L) and tetrabutylammonium (5mmol/L). The VFA quantified were acetate, propionate, butyrate, valerate, formate, lactate and caproate. Ammonium (NH<sub>4</sub><sup>+</sup>) concentration was measured using the Nessler's colorimetric method following the French standard (NF T 90-105) in spectroscopic tanks using Hach spectrometer DR2800. The link between Free Ammonia Nitrogen (FAN), Total Ammonia Nitrogen (TAN), pH and temperature can be summarized with the following equation (Anthonisen et al., 1976):

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

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 was extracted using the commercial kit FastRNA Pro<sup>TM</sup> Soil-Direct (MP Biomedicals) 189 following the manufacturer's specifications. Then, DNA co-extracted was removed using 190 TURBO<sup>™</sup> DNase (Ambion) kit following the manufacturer's instructions. The RNA was 191 denaturated by 2 min at 85°C in a dry bath and was then stored on ice. RNA purification was 192 carried out using the Agencourt AMpure RNA magnetic beads purification system (Beckman 193 Coulter) by adding 1.8 volumes of beads by volume of RNA. After mixing by pipetting and 5 194 min of incubation, beads were captured using a magnetic rack on one side of the tube and then 195 196 washed by adding 500µL of 70% cold ethanol (diluted in DEPC-water). After incubation of the tube during 30 seconds at room temperature, the ethanol was removed. This washing step was repeated 3 times. Once ethanol finally evaporated, beads were resuspended with DEPCwater to eluted RNA from the beads. Finally beads were removed using the magnetic rack and RNA was recovered in the supernatant. The integrity and quantity of the RNA was evaluated using the Hight Sensitivity RNA ScreenTape and 4200 TapeStation (Agilent Technologies) following the manufacturer's protocol.

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

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

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

216

217 3. Results and discussion

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

The physico-chemical characteristics of the different substrates and the inoculum are summarised in Table 1. The C/N ratio of fish was lower than the C/N ratio of sludge and grass which were similar. Two key information can be drawn from these analyses. The first one is the higher amount of nitrogen (N) in fish waste (9.5 %) compared to sludge and grass (2.46 and 2.2 % respectively), explaining the low C/N ratio. This result implies a higher potential to produce ammonia during the fish degradation compared to sludge and grass. The second information is the lower quantity of dissolved organic carbon in sludge (1250 mgC/L) compared to fish (7921 mgC/L) and grass (7692 mgC/L). It suggests that even if the C/N ratio was similar between grass and sludge, the CH<sub>4</sub> production could start earlier in fish and grass because more readily available carbon was present.

## 229 [[insert table 1]]

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

239 [[insert table 2]]

240 3.2. Mono-digestion of the substrates

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

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

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

# 272 [[insert figure 1]]

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

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

The evolution of  $NH_4^+$ , pH and  $NH_3$  values during the substrates degradation are 299 presented in the supplementary figure B.1-C. F-100 produced in 7 days around 1300 mg/L of 300 NH<sub>4</sub><sup>+</sup> compared with 350 mg/L for G-100 and S-100 corresponding to a respectively amount 301 of 140 and 30 mg/L of NH<sub>3</sub>. Ammonia nitrogen is known to be an inhibitor of the AD, 302 especially free ammonia (NH<sub>3</sub>) (Fotidis et al., 2013; Rajagopal et al., 2013). However a wide 303 304 range of half inhibitory concentrations has been reported between 1.7 to 19 g/L of  $NH_4^+$ (Chen et al., 2008; Poirier et al., 2016) and 50-1400 mgNH<sub>3</sub>/L (Rajagopal et al., 2013) 305 306 depending on multiple factors such as the microbial community, temperature... The highest free ammonia accumulation observed for F-100 was 140 mg/L at day 50. The amount of 307 ammonia observed in F-100 was under the inhibitory values described in the literature. It 308 309 cannot be excluded that the microbial community was partly inhibited, particularly the methanogens and the propionate degrading acetogenic bacteria which are known to be 310 311 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

Evolution of the cumulated CH<sub>4</sub> production over time for the different mixtures is presented in Figure 2 and the Table 2 details the results of the Gompertz modelling for each mixture. In all cases the addition of fish enabled to increase the final CH<sub>4</sub> production compared to S-100. For example, F-25, which contains 25 % of fish and 75 % of sludge, produced 65.58 mgC/gCOD of CH<sub>4</sub>, *ie*. 18 mgC/gCOD more than S-100. According to the Gompertz modelling the latency before CH<sub>4</sub> production start was not significantly modified between the different mixtures, but the production rate was increased from 2.31 to 3.00 mgC/D/gCOD for F-25 and F-75 respectively. Dissolved organic carbon accumulation (supplementary figure C.1-A) between days 0 to 7, representative of the solid carbon degradation during the early hydrolytic phase, increased when more than 25 % (gCOD) of fish was mixed to WAS. This could be explained by the presence of a higher quantity of easily degradable carbon or by a hydrolysis step faster when fish was present than for S-100.

Volatile fatty acids accumulation, presented in the supplementary figure C.1-B show a 327 similar VFA pattern evolution for the mixtures F-25 and F-50 than for S-100. The acetate 328 329 accumulation profile in F-75 was also similar to S-100 while the propionate maximum accumulation was closed to F-100 (45 mgC/L/gCOD). The consumption of the propionate in 330 331 F-75 was completely achieved after day 60 while in F-100 the propionate degradation had not started yet. Regarding the butyrate production no significant effect of AcoD was observed. 332 The ammonia accumulation presented in the supplementary figure C.1-C shows an increase of 333 334 the NH<sub>4</sub><sup>+</sup> level proportional to the amount of fish added in the feeding, indicating that ammonia production was mainly due to fish. Use wastewater sludge to dilute fish waste 335 decreased the NH<sub>4</sub><sup>+</sup> level which seemed to reduce the inhibition of the propionate degrading 336 population observed on the fish mono-digestion. This allowed to increase the propionate 337 degradation rate and to produce the CH<sub>4</sub> faster. 338

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

In the case of co-digestion with grass, the final  $CH_4$  production was increased only when more than 50 % of grass was mixed with sludge compared to sludge mono-digestion (Fig. 2). G-25 presented an earlier  $CH_4$  production but a final production similar to S-100. The more the proportion of grass was important the more the latency to produce  $CH_4$  was reduced (from 4.5 days to 2.6 for G-25 and G-75 respectively). Mixing grass and sludge increased the amount of dissolved organic carbon accumulated during the 7 first days. All the mixtures presented the same evolution of acetate accumulation than G-100 with a higher and faster accumulation than in S-100. However, propionate final accumulation was 2 times higher for the mixtures than for S-100 (20 mgC/L/gCOD) and G-100 (37 mgC/L/gCOD) but propionate degradation for all bioreactors was achieved in 30 days. For all bioreactors of grass co-digestion, the ammonia level stayed stable along the experiment closed to 350mg/L such 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

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

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

In the fish mono-digestion the gas production at the beginning of the experiment was 362 363 dominated by the acetoclastic pathway. During the first week of the experiment, the methanogenic pathway increased from 1.04 to 1.06, namely from acetoclastic pathway to a 364 mix of methanogenic pathways. This can be explained by a high hydrolytic activity producing 365 366 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 methanogens. Finally, when all the acetate was degraded, the  $\alpha_{app}$  increased again due to the 368 369 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 the mixtures of the co-digestion with fish followed the same evolution than F-100 but with the 371

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

The mono-digestion of grass presented a similar evolution across time of the 376 methanogenic pathway. The beginning of the experiment started in the acetoclastic pathway. 377 378 After a first increase followed by a decrease of the  $\alpha_{app}$  induced by the production of CO<sub>2</sub> then the consumption of the acetate, the  $\alpha_{app}$  stabilised at 1.055 due to the propionate degradation. 379 Contrary to the fish mixtures, the co-digestion mixtures of grass presented a distinction 380 381 between two groups. The methanogenic pathway evolution for G-25 was closer to S-100 with a beginning in the hydrogenotrophic pathway while the methanogenic pathways evolution of 382 G-50 and G-75 was similar to G-100. This differentiation in the methanogenic pathways for 383 384 the grass mixtures can explain the difference observed in the methane production.

385 [[insert figure 3]]

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

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

# 401 [[insert figure 4]]

402

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

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

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

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

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

was increased by 1.7 times compared to the mono-digestion of wastewater of sludge and 0.9compared 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 improved the final methane production up to 1.9 times when 75 % of fish was added. On the 452 contrary grass enabled to improve the final methane production from WAS only when more 453 than 25 % of grass was added. Adding more than 50 % of grass improved both the production 454 455 rate and the final production by to 1.5 and 1.7 times respectively. Specifically built indicators showed that using 75 % of fish or grass as co-substrate with sewage sludge enabled to obtain 456 the maximum final methane production. In nearly all the bioreactors, archaea from 457 Methanosarcina genus accounted for more than 75% of the archaeal diversity. No significant 458 difference in the methanogenic pathways was observed across time between fish and grass 459 mono-digestion. It was mostly acetoclastic while wastewater sludge mono-digestion changed 460 461 from hydrogenotrophic to acetoclastic methanogenesis pathway. The anaerobic co-digestion allowed to limit the variation between the methanogenesis pathway of the sludge. 462

463

# 464 Acknowledgements

We want to thank Lénaïck Rouillac and Nadine Derlet from the Irstea PROSE analytical
division for their technical support. Our acknowledgments also go to SUEZ Environment for
providing us access to the wastewater treatment plant of Valenton.

468

## 469 Funding

470	This work was supported by the National Research Agency (ANR-16-CE05-0014).
471	The funders had no role in study design, data collection and analysis, decision to publish, or
472	preparation of the manuscript.

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	Wastewater	Fish	Grass	Incoulum	
	Sludge			moculum	
$NH_4^+$ (mgN/L)	299	899	438	628	
DOC (mgC/L)	1250	7921	7692	149	
DIC(mgC/L)	99	346	424	753	
COD (gO2/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	

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

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.

	Name	$\mu$ (mgC/day/gCOD)	λ (day)	A (mgC/gCOD)
	F-100	2.35 (±0.57)	10.1 (±0.8)	87.55 (±13.00)
SU	F-75	3.00 (±0.11)	10.8 (±0.6)	86.31 (±4.93)
atcl	F-50	2.49 (±0.35)	7.7 (±0.8)	72.03 (±4.49)
al B	F-25	2.31 (±0.10)	9.4 (±0.5)	64.65 (±1.46)
enta	S-100	2.26 (±0.26)	12.8 (±1.3)	46.62 (±0.8)
rim	G-25	1.93 (±0.10)	4.5 (±0.6)	42.75 (±5.41)
kpei	G-50	2.97 (±0.21)	3.2 (±1.1)	70.80 (±4.72)
Ð	G-75	3.45 (±0.46)	2.6 (±0.7)	79.30 (±6.99)
	G-100	2.81 (±0.12)	3.1 (±0.2)	68.43 (±0.97)
a	F75-MDI	2.33 (±0.49)	10.8 (±0.9)	77.32 (±9.95)
stio	F50-MDI	2.31 (±0.43)	11.5 (±1.1)	67.09 (±6.90)
)ige: cato	F25-MDI	2.28 (±0.35)	12.1 (±0.5)	56.85 (±3.85)
0-D ndic	G25-MDI	2.40 (±0.24)	10.4 (±1.0)	52.07 (±0.84)
10n I	G50-MDI	2.54 (±0.20)	8.0 (±0.8)	57.53 (±0.89)
4	G75-MDI	2.67 (±0.16)	5.5 (±0.5)	62.98 (±0.93)
est	Fish-BMP	8.18 (±0.24)	0	119.35 (±15.28)
IP (	Sludge-BMP	2.09 (±0.17)	0	44.35 (±18.3)
BN	Grass-BMP	8.47 (±3.67)	0	118.33 (±12.9)
uo	F75-ODI	6.66 (±0.22)	0	100.60 (±16.04)
esti r	F50-ODI	5.14 (±0.21)	0	81.85 (±16.79)
Dig cato	F25-ODI	3.61 (±0.19)	0	63.10 (±17.55)
nal	G25-ODI	6.88 (±2.80)	0	99.84 (±14.25)
ptin L	G50-ODI	5.28 (±1.92)	0	81.34 (±15.60)
Ō	G75-ODI	3.69 (±1.05)	0	62.85 (±16.95)



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



Figure 2. Cumulated CH<sub>4</sub> production (mgC/gCOD) over time (days) for Fish and Grass used as co-substrates in co-digestion with wastewater sludge. Mean values of the triplicate bioreactors, error bars represent standard deviation within triplicates. S-100 stands for wastewater sludge alone, F-25, F-50, F-75, F-100 stands for respectively 25, 50, 75 or 100% of fish (F) in co-digestion with sludge, G-25, G-50, G-75, G-100 stands for respectively 25, 50, 75 or 100% of Grass (G) in co-digestion with sludge.



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



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



656

Figure 5. Experimental Batch methane production (mgC/gCOD) compared to the Maximal Digestion Indicator and the Optimal Digestion Indicator over time (days) for 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.
664	