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1 Assessment of Substrate Biodegradability Improvement in Anaerobic
2 Co-digestion using a chemometrics-based Metabolomic Approach
3

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15 **Declarations of interest:** none

16

17 **Abstract:**

18 Anaerobic co-digestion (AcoD) can increase methane production of anaerobic digesters
19 in plants treating wastewater sludge by improving the nutrient balance needed for the
20 microorganisms to grow in the digesters, resulting in a faster process stabilization. Substrate
21 mixture proportions are usually optimized in terms of biogas production, while the metabolic
22 biodegradability of the whole mixture is neglected in this optimisation. In this aim, we
23 developed a strategy to assess AcoD using metabolomics data. This strategy was explored in
24 two different systems. Specifically, we investigated the co-digestion of wastewater sludge with
25 different proportions of either grass or fish waste using untargeted High Performance Liquid
26 Chromatography coupled to Mass Spectrometry (HPLC-MS) metabolomics and chemometrics
27 methods. The analysis of these data revealed that adding grass waste did not improve the
28 metabolic biodegradability of wastewater sludge. Conversely, a synergistic effect in the

29 metabolic biodegradability was observed when fish waste was used, this effect being the highest
30 for 25% of fish waste. In conclusion, metabolomics can be regarded as a promising tool both
31 for characterizing the biochemical processes occurring during anaerobic digestion, and for
32 providing a better understanding of the anaerobic digestion processes.

33

34 **Keywords: metabolomics, anaerobic digester, co-digestion, methanization, HPLC-MS,**
35 **CCA**

36

37 **1. Introduction:**

38 Anaerobic digestion (AD) is a sustainable multistep process for the treatment of organic
39 waste used to reduce the amount of solid organic matter resulting from the management of
40 wastewater, and in consequence, the costs for waste handling. Moreover, this process generates
41 renewable energy in the form of biogas by the action of the microorganisms digesting the
42 organic matter (Madigou et al., 2019).

43 Biogas production in anaerobic digesters relies strongly on the stability of the microbial
44 community growing in the anaerobic digesters (Calusinska et al., 2018). One key parameter is
45 the carbon-to-nitrogen (C/N) ratio. Low C/N ratios, found for example in wastewater sludge,
46 can result in low digestion rates and low biogas production (Li et al., 2011). To increase the
47 C/N ratio and stimulate sludge digestion, the waste is digested in combination with other
48 substrates richer in carbon. This approach is known as anaerobic co-digestion (AcoD), and is
49 considered to be very ecologically efficient since several types of waste can be processed
50 simultaneously (Borowski and Kubacki, 2015). In this work, we have explored the effect of
51 mixing wastewater sludge (WAS) with either garden grass (GG) or fish waste (FW) as co-
52 substrates.

53 In the digestion of the organic waste, the complex polymers (carbohydrates, proteins,
54 nucleic acids and fats) are broken down into simpler compounds (sugars, amino acids, nucleic

55 bases, glycerol and fatty acids) that are ultimately converted to methane and CO₂ by the
56 microbial community living in the ADs (Chatterjee and Mazumder, 2019; Meegoda et al.,
57 2018). Despite the metabolic course of the AD, the evolution of the digestion is typically
58 assessed with indirect measurements of the digesters performance (i.e., pH and biochemical
59 oxygen demand (Meegoda et al., 2018)), or by studying the microbial dynamics by RNA and
60 DNA sequencing (De Vrieze et al., 2018). Alternatively, specific metabolites or regulatory
61 compounds can also be analyzed to look for underlying metabolic mechanisms
62 (Vanwonterghem et al., 2014). Lastly, to our knowledge, only a few studies have employed
63 metabolomic approaches to explore the global metabolic dynamics in ADs (Beale et al., 2016;
64 Murovec et al., 2018; Yang et al., 2014).

65 Metabolomics is the study of small molecules (metabolites) within cell extracts, tissues
66 and living organisms (Patti et al., 2012), and has been used extensively to evaluate the dynamic
67 metabolic response of living systems to physiopathological stimuli and genetic modifications
68 (Klassen et al., 2017). Metabolomics has also been used to study the metabolites from microbial
69 communities (the so-called “community metabolomics”) (Llewellyn et al., 2015). Community
70 metabolomics studies concerning AD are still very scarce (Jones et al., 2016), since its
71 application has been challenging due to the very large range of metabolites for which there is
72 limited *a priori* knowledge (Vanwonterghem et al., 2014). To reduce the complexity of this
73 analysis, the community metabolomics studies of ADs are focused on either the solid fraction
74 (sludge) (Beale et al., 2016) or on the liquid fraction (digestate) (Murovec et al., 2018).
75 Metabolomics data analyses in these two studies were exploratory and despite they showed the
76 suitability of metabolomics to characterize AD samples, the application interest of these studies
77 was not demonstrated.

78 In this line, the two objectives of the present work are 1) to identify the metabolites that
79 can be used to assess the GG and FW co-digestion of sludge, and 2) to identify the optimal
80 proportion of GG or FW co-substrate that maximizes WAS digestion.

81 To achieve these goals, some considerations were taken into account beforehand. First,
82 we have only analyzed the digestate to focus on the extracellular digestion products, as the
83 metabolomic characterization of sludge would also include the intracellular metabolites from
84 the microorganisms growing in the digesters. Second, a time-course experimental design was
85 used to capture the main metabolomic dynamics over time. And third, samples were measured
86 using HPLC-MS and further explored with untargeted chemometrics approaches (Principal
87 Components Analysis (PCA) and Common Components Analysis (CCA)) (Bouhleb et al., 2018;
88 Martin et al., 2015) to unravel the underlying metabolomic dynamics occurring in the studied
89 ADs. In sum, this study provides a novel methodological approach to characterize the
90 metabolomic processes in ADs that can be used to assess digester efficiency while at the same
91 time expanding knowledge in this new field of community metabolomics.

92

93 **2. Methods**

94 2.1. Feedstock preparation and experimental set-up

95 Wastewater sludge (WAS) was collected from an industrial wastewater treatment plant
96 (Valenton, France), GG was from the IRSTEA Institute's lawn, and FW was obtained from a
97 fish market. GG and FW were crushed separately and the resulting minced solids were stored
98 at 4°C for two days. The chemical characteristics of WAS, FW and GG are given in **Table A.1**.

99 The inoculum was obtained from a mesophilic full-scale anaerobic digester treating
100 primary sludge at the Valenton (France) wastewater treatment plant. To prepare the inoculum
101 before use, it was left under anaerobic conditions at 35°C for two weeks to digest the residual
102 organic matter remaining in the inoculum. For the experimental set-up, 27 anaerobic batch

103 bioreactors consisting of 1 L glass bottles were used. Nine different mixtures were prepared by
104 blending WAS substrate with FW or GG co-substrates in different proportions (100% WAS,
105 75% WAS/25% GG, 50% WAS/50% GG, 25% WAS/75% GG, 0% WAS/100% GG, 75%
106 WAS/25% FW, 50% WAS/50% FW, 25% WAS/75% FW, and 0% WAS/100% FW) of grams
107 of Chemical Oxygen Demand (gCOD). Each type of substrate mixture was used in three
108 reactors (triplicates). In all bioreactors, the amount of added biomass was fixed at 12 gCOD.
109 Then, all 27 bioreactors were inoculated with 1.2 gCOD of anaerobic sludge. All the digesters
110 were complemented with a biochemical potential buffer (International Standard ISO 11734
111 (1995) (International Organization for Standardization, Geneva, 1995)) up to a final volume of
112 700 mL. Bioreactors were sealed with a screw cap and a rubber septum and headspaces were
113 flushed with N₂ (purity > 99.99 %, Linde gas SA), and incubated for 4 weeks at 35°C in the
114 dark without agitation.

115 2.2. Sampling

116 A particular sampling experimental design was used to focus on the metabolic
117 fingerprint associated with the biogas production. For every digester containing either FW or
118 GG, three points were monitored: one at the start of the co-digestion (day 0), and the two
119 samples closest in time (before and after) to the largest methane production (days 14 and 21 for
120 GG digesters, and days 21 and 28 for FW digesters) (Cardona et al., 2019). Finally, for digesters
121 containing only sludge, all 4 time-points were monitored (days 0, 14, 21, and 28). Considering
122 the triplicates, in total, 84 samples were collected. Sampling was performed by collecting 6 mL
123 of liquid phase from the digester through the septum using a syringe. Then, samples were
124 centrifuged at 10,000 g for 10 minutes to collect the supernatants, which were then snap frozen
125 in liquid nitrogen and kept at -20°C prior to metabolomic analysis.

126 2.3. Metabolomic analysis

127 Metabolomic analysis was performed on all collected supernatants. Prior to injection,
128 samples were diluted to 1/10 in water. Instrumentation consisted in an Accela 1250 pump
129 system connected to a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, MA, US)
130 operated in positive electrospray ionization mode (ESI+). The detection was performed in full
131 scan over an m/z range from 50 to 500 at a resolution of 100,000. The analytical column was a
132 50×2.1 mm inner diameter, $1.7 \mu\text{m}$ Synchronis C18 (Thermo Scientific, MA, US). The two
133 mobile phases were acetonitrile 0.05% formic acid (phase A) and H_2O 0.05% formic acid
134 (phase B). For each sample, $10 \mu\text{L}$ were injected into the analytical system. The flow rate was
135 set at 0.4 mL min^{-1} , and the chromatographic method consisted of a linear gradient of A/B
136 solvents changing from 10:90 to 80:20 over 23 minutes, followed by a stabilization phase of 5
137 minutes to return to the initial condition.

138 To remove possible batch effects, samples were injected in random order. Moreover,
139 Quality Controls (QCs) composed of a pool of all samples were injected every 5 samples, and
140 blank samples were injected every 10 samples. In total, 116 samples were injected (84
141 experimental samples, 21 QC samples, and 11 blank samples).

142 2.4 HPLC-MS data preprocessing

143 Raw HPLC-MS data were converted into mzXML-format files using MSConvert
144 (ProteoWizard 3.0). Then, the list of chromatographic features (regions of interest, or ROIs)
145 from each sample were extracted using the XCMS R-package (version 1.52.0) (Smith et al.,
146 2006). ROIs were generated with the method *centWave*, using a m/z error threshold of 10 ppm
147 and a peakwidth between 20 and 50 seconds. ROIs found in different samples were grouped
148 using the *group* method with a bandwidth of 30. ROI retention times from the same ROI groups
149 were unified across samples using the *obiwarp* method (Prince and Marcotte, 2006). A second
150 grouping was carried out using a bandwidth of 25. Then, samples with missing ROIs were filled

151 using the *fillPeaks* method. From this analysis, a table of peak intensities composed of 116 rows
152 (one per sample) and 476 columns (one per ROI) was obtained. This table was then imported
153 as a matrix into Matlab R2009b (Mathworks, Inc., MA, US) for intensity drift and batch
154 corrections. Drifts in signal intensity were corrected using LOESS (Rusilowicz et al., 2016;
155 Zelena et al., 2009). On the other hand, differences within batches (between triplicates) and
156 between batches (different substrate mixtures and time-points) were minimized with the PQN
157 normalization method (Frank Dieterle et al., 2006), using the same approach as in Puig-
158 Castellví (Puig-Castellví et al., 2016).

159

160 2.5. Chemometric data analysis

161 First, the matrix composed of the metabolomics data from the 84 experimental samples
162 was auto-scaled (van den Berg et al., 2006) and investigated by Principal Components Analysis
163 (PCA (Bro and Smilde, 2014)). With this method, a preliminary overview of the data was
164 obtained.

165 With the aim of identifying the underlying metabolomic processes that govern the
166 different biological dynamics occurring in the GG- and in the FW-containing digesters, the data
167 were arranged into two matrices (one per co-substrate). Samples included in the first matrix
168 were obtained from the two mono-digestions (WAS and GG), and from the co-digestion of
169 WAS and GG, and collected at days 0, 14 or 21. Samples in the second matrix were from the
170 analogous mono- and co-digestions of WAS and FW. In this second case, samples were
171 collected at days 0, 21 and 28. Thus, each matrix is composed of 45 samples (3 samples per
172 condition and per time, with 5 substrate conditions and 3 time-points). These two matrices will
173 be referred to as ‘GG dataset’ and ‘FW dataset’, respectively.

174 Each dataset was centred and norm-scaled (Bylesjö et al., 2009) and investigated by
175 Common Components Analysis (CCA) (Bouhleb et al., 2018; Rutledge, 2018). CCA is an

176 unsupervised chemometric method that estimates a series of orthogonal common components
177 (CCs) that are linear combinations of the original variables, with strong weightings for strongly
178 correlated variables that present the same dispersion of the observations. Analogously to PCA,
179 for every CC, scores and loadings vector are calculated. Loadings give information about the
180 metabolic profile (or metabolite composition) of the analyzed samples, while scores show the
181 relative importance of these metabolic profiles for every sample. Besides, in CCA, a vector of
182 weights (called saliences) corresponding to the importance of the variables for each CC is also
183 iteratively calculated. The optimal number of CCs can be assessed by plotting the sum of
184 saliences for each component, in the same manner as the assessment of the optimal number of
185 components in a PCA with the Scree test (Ledesma et al., 2015) or by applying the Kaiser-
186 Meyer-Olkin Measure of Sampling Adequacy (KMO) to the residuals matrices to detect the
187 moment when their lines and/or columns are no longer sufficiently correlated to merit further
188 factorial analysis (Rencher, 2002).

189 For every CC, variables (*e.g.*, ROIs) associated with loading values beyond 2 standard
190 deviations ($\pm 2 \times SD$) were selected (Bouhlef et al., 2018) and tentatively assigned if possible.

191 2.6 Metabolite identification

192 Tentative metabolite identification of the selected ROIs was performed based on the
193 comparison of the accurate molecular mass measured by HPLC-MS with the calculated exact
194 mass. Molecular formula of candidates were first estimated by using the *Rdisop* R-package
195 (Böcker et al., 2009). This package allows the calculation of all the possible molecular formulae
196 for a given accurate mass, within a delta error window in ppm (difference between the
197 experimental mass and the adduct mass), and using a limited number of elements. In this
198 analysis, we sought all the molecular formulae within 10 ppm that matched with
199 $C_cH_hN_nO_oP_pS_sNa_{na}K_kCa_{ca}$. Then, for every accurate molecular mass, proposed molecular
200 formulae were filtered using heuristics (Kind and Fiehn, 2007), and finally, the molecular

201 formula associated to the lowest delta value was selected. Finally, compound names were given
202 to the selected molecular formulae after inspection of the possible structures in HMDB (Wishart
203 et al., 2018), LipidMaps (Fahy et al., 2007), and PubChem (Kim et al., 2019) compound
204 libraries.

205 2.7 Assessment of metabolic degradability during AcoD

206 In this paper, we propose to quantify the metabolic biodegradability (MB) of the different
207 studied mixtures to assess the improvement in the AcoD performance compared to the
208 corresponding mono-digestion experiment.

209 $MB_{0 \rightarrow i}$ is defined as the observed change in the overall metabolite composition during AD
210 between the initial time-point (t_0) and t_i . Since CC scores represent the relative importance of
211 the distinct metabolic profiles, MB can be approximated as the distance between the two sample
212 points in a scores plot obtained from a CCA analysis of a metabolomics dataset. For a CCA
213 model of two components (CC1 and CC2), this expression can be generalized as eq. (1):

$$214 \quad MB_{s,0 \rightarrow i} = \sqrt{(u_{CC1,s,t_i} - u_{CC1,s,t_0})^2 + (u_{CC2,s,t_i} - u_{CC2,s,t_0})^2} \quad \text{eq. (1)}$$

215 In eq. (1), u_{CC1,s,t_0} and u_{CC1,s,t_i} represent the CC1 scores values associated to a given substrate
216 composition s at the two time-points, t_0 and t_i ; and u_{CC2,s,t_0} and u_{CC2,s,t_i} represent the
217 corresponding CC2 scores values for the same substrate composition and times. As graphical
218 examples, the $MB_{0 \rightarrow i}$ for 50% GG, 100% GG, 50% FW, and 100% FW are represented with
219 arrows in **Fig. 2.**

220 Then, the metabolic biodegradability rate (MBR) can be calculated using eq. (2) as follows:

$$221 \quad MBR_{\text{substrate_mixture}} (\%) = \frac{MB_{\text{substrate_mixture}}}{MB_{\text{pure co-substrate}}} * 100, \quad \text{eq. (2)}$$

222 where $MB_{\text{substrate_mixture}}$ is the MB value obtained for every tested substrate, and $MB_{\text{pure co-substrate}}$
223 is the MB value for the pure co-substrates (in this study, 100%GG or 100%FW depending on

224 the studied AcoD system) at the latest time-point, as the latter is presumed to have the highest
225 observed MB.

226 Finally, the factor of synergy in the co-digestion (FSC) can be estimated from the MBR. The
227 other hand, FSC is the ratio between the observed and the theoretical MBR for a particular co-
228 digestion mixture:

$$229 \text{ FSC}_{\text{substrate mixture}} = \frac{\text{MBR}_{\text{substrate mixture}}}{\text{MBR}_{\text{theoretical}}}, \quad \text{eq. (3)}$$

230 where the theoretical MBR is the weighted sum of the MBR from the substrate and the co-
231 substrate by their corresponding ω_s relative proportions:

$$232 \text{ MBR}_{\text{theoretical}} = \sum_{s=1}^{s=S} \omega_s \text{ MBR}_s, \quad \text{eq. (5)}$$

233 For instance, if 100% WAS has a MBR of 20% and 100% GG has a MBR of 100%, then the
234 theoretical MBR of 50% WAS/50% GG will be of 60%.

235 A FSC of 1 indicates that the MBR of the studied CoD is equivalent to the MBR obtained by
236 digesting the two substrates separately. A FSC above 1 means that a synergistic effect in the
237 MBR occurred due to the CoD, while a FSC below 1 reflects a loss of efficiency in the metabolic
238 degradation due to the CoD.

239

240 **3. Results and Discussion**

241 Detection and identification of metabolites from bio-waste matter is a challenging task,
242 since knowledge about their chemical composition is limited (and especially for WAS (Alves
243 Filho et al., 2015; Vanwonterghem et al., 2014)). Moreover, the metabolic range of detected
244 metabolites can be wide, as it may include not only the metabolites intrinsic to the three
245 substrates (WAS, GG, and FW), but also those metabolites resulting from the degradation of
246 these substrates. With the aim of addressing this challenge, we have taken advantage of both

247 the resolution and sensitivity of HPLC-MS, and of advanced chemometric methods capable of
248 untangling the complex mixtures being analyzed.

249 3.1 Unsupervised analysis of the metabolomics dataset

250 After the analysis of the ADs samples with HPLC-MS, the acquired data were
251 preprocessed and analyzed with *XCMS* R-package (see sections 2.3 and 2.4 in methods). With
252 this methodology, a total of 476 features per sample were obtained.

253 The resulting data were arranged into a matrix consisting of 84 rows (samples) and 476
254 columns (features). The data matrix was first analyzed with PCA to investigate its data variance.
255 The first three PCs only captured 32.02% of the dataset variance, revealing a complex
256 underlying data structure. The first component (14.14% of the variance) separated samples
257 collected at the first time-point from the rest, PC2 (10.67%) separated samples from the two
258 co-substrates (grass and fish waste), and PC3 (7.21%) separated samples according to WAS
259 relative composition (**Fig. 1**). The observed scores distribution for the other components could
260 not be satisfactorily linked to any studied factor (proportion of co-substrate or time).

261 From this initial analysis, it is observed that the major difference between the samples
262 is due to time, specifically between the first (square) and second (triangle or circle, depending
263 on the co-substrate) time-points (PC1 in **Fig. 1A**). This can be interpreted as follows: there
264 exists a set of metabolites, present in all samples regardless of the nature of the substrate, which
265 are rapidly consumed by the microbial communities before the second time-point. After these
266 metabolites are consumed, the metabolism of the microbial communities shifts to consume the
267 less assimilable metabolites. This process is less efficient and much slower, and it continues
268 until the end of the experiment.

269 As a second observation, during the course of the experiment, the most important
270 metabolic changes are produced in the reactors containing a greater amount of co-substrate

271 **(Fig. 1A)**. This results in the clusters of samples containing co-substrate being much larger than
272 the cluster of 100% WAS samples (the orange cluster in **Fig. 1A**). This suggests that the most
273 biodegradable compounds are found in GG and FW substrate rather than in WAS.

274 3.2 Common Components Analysis (CCA)

275 In a previous work (Cardona et al., 2019), it was concluded that the use of FW caused a
276 delay in the performance compared to GG-containing digesters. In order to cope with this
277 difference in delays, different time-points were monitored depending on the type of co-substrate
278 used. Thus, after observing that both GG- and FW-containing digesters presented independent
279 trends in terms of performance and metabolomic profiles (as seen in **Fig. 1B**), to simplify the
280 interpretation of the metabolomics data, these two sets of digesters were analyzed as two
281 independent experiments.

282 In order to unravel the metabolic processes occurring in GG- and FW-containing
283 anaerobic digesters, GG and FW datasets were further explored with CCA analysis. With CCA,
284 metabolites presenting the same dispersion of the set of samples (and therefore descriptive of
285 the same underlying biological process) are grouped together in a series of CC components that
286 are a linear combination of the original metabolites, and where the most significant metabolites
287 (see *Methods*) are considered to be representative of the metabolomic fingerprint of the
288 digesters.

289 The Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO) showed that there
290 were only two components that meaningfully describe each dataset, the other components
291 extracting only noise; as they could not be linked to substrate composition nor to time. In other
292 words, the changes in the metabolite concentrations observed over time across reactors were
293 associated with two main metabolic processes (one per CC). CC scores are presented in **Fig. 2**.

294 Regarding the scores obtained for the analysis of the two datasets, CC1 and CC2 (**Fig.**
295 **2**) are descriptive of metabolites from the co-substrate. This can be seen since 100% WAS
296 samples remained clustered together regardless of the collection time, and the most prominent
297 changes over time occurred in the digesters containing the highest amount of co-substrate.

298 Interestingly, the score distributions for both AcoD systems are triangle-shaped (drawn
299 using dashed grey lines in **Fig. 2**). In these two triangles, the three vertices correspond to (1)
300 the 100% WAS samples, (2) the 100% co-substrate samples at the initial time-point, and (3) the
301 same samples at the last time-point. This particular score distribution is observed due to two
302 reasons. First, because we used an experimental design to screen the degradability of the
303 mixtures at different proportions. And second, because the largest variability in the studied
304 system is related to the degradation of the co-substrate and the lowest one to WAS. Hence, due
305 to the particularities of AcoD, this score distribution should be also expected for any other
306 proportion-based experimental design of a AcoD system using organic waste as co-substrate.

307 In both GG and FW AcoD systems, CC1 components scores sign change (from positive
308 to negative) between the first and the second time-point, implying that either metabolites have
309 been consumed or produced after the first time-point. In addition, the small differences
310 observed between the scores from the second and the third time-point indicate that CC1 only
311 describes the metabolic alterations from the earlier stage. So, this component is representative
312 of the most rapidly consumed metabolites from the co-substrate, and also of the products
313 resulting from this fast metabolic process. On another hand, CC2 separates digesters rich in co-
314 substrate (positive scores) from those with a lower amount of co-substrate (negative scores).
315 For both GG and FW AcoD systems, the metabolic changes over time in CC2 were more
316 important for digesters rich in co-substrate.

317 3.3 Feature selection

318 For the GG dataset, 35 features were selected for CC1 and 41 for CC2 while for the FW
319 dataset, 18 features were found to be representative for CC1 and 17 for CC2 (**Figure 3**). The
320 lower number of relevant features for the FW dataset could be the result of the smaller number
321 of metabolites in fish than in grass (due to the existence of the plant secondary metabolite in
322 the latter (Fiehn, 2002))-

323 Spectral features assigned to metabolites are shown in **Table. A.2** (for GG dataset) and
324 **Table. A.3** (for FW dataset). All metabolites represented by CC1 (**Fig3A** and **Fig3B**) are
325 associated with positive loadings, and therefore correspond to metabolites being consumed over
326 time. On the other hand, metabolites represented by CC2 (**Fig3C** and **Fig3D**) are associated
327 with either positive or negative loadings, indicating that these components are descriptive of a
328 more complex metabolomic dynamic that is less influenced by time and more dependent on the
329 co-substrate mixture, as commented in section 3.2.

330 3.4 Metabolomic fingerprint of the GG anaerobic digesters

331 In GG digesters, CC1 is mainly descriptive of the breakdown of the proteins from grass
332 and the consumption of the products obtained in this process. Related to this process, CC1
333 includes 7 dipeptides, 4 aminoacids, one acetylated amino acid, and 7 compounds from amino
334 acid catabolism (**Table. S1**). This result is in agreement with the first phase of the anaerobic
335 digestion, also known as the hydrolytic phase. In a previous metabolic study of the ADs of
336 vegetal matter, it was observed that the different phases of the anaerobic digestion give distinct
337 metabolomic signatures (Yang et al., 2014).

338 Apart from the protein breakdown and assimilation, the CC1 component is also
339 descriptive of the degradation of nucleic bases and nucleotides. Detected degradation products
340 from this pathway are xanthine, beta-alanine, and nicotinic acid (**Table. A.2**).

341 Some plant-specific metabolites were also included in CC1, such as D-mannitol and an
342 acetamide compound. . Acetamides are produced during fermentation processes of plant matter
343 (Linskens and Jackson, 1988). Finally, the detection of two sulfide compounds (dipropyl sulfide
344 and ethylpropyl disulfide) can be regarded as a signature of the hydrogen sulfide commonly
345 generated during microbial degradation processes (Zerrouki et al., 2015).

346 The metabolomic profile for CC2 from the GG dataset is mainly descriptive of oxidized
347 free fatty acids (11 features). The reduced forms of these lipids may be grass constituents (as
348 they are associated with positive loadings), and they may have been oxidized after cell walls
349 were disrupted during the digestion process. In the same line, a fatty aldehyde was detected and
350 linked to grass substrate in this component. In addition, a polyphenol was found specific for
351 this component.

352 Conversely, there are only 4 features in CC2 that are characteristic of WAS. 3 of these
353 features (X257, X273, and X306 in **Table A.2**) were assigned to small compounds containing
354 heteroatoms (such as phosphorous (Alves Filho et al., 2015) and sulfur (Du and Parker, 2013))
355 and to unsaturations. The relative structural simplicity of these compounds may indicate that
356 they have been generated in the degradation of more complex organic compounds originally
357 found in the WAS in earlier digestion stages (i.e., during the microbial aerobic digestion). The
358 other compound was assigned to a secosteroid. Secosteroids are formed after ring cleavage of
359 sterol lipids, which may have occurred by enzymatic reaction during the anaerobic digestion.
360 The presence of secosteroids in the WAS substrate may also be the result of the partial digestion
361 of steroids from sewage waste. These compounds may come from the dissolved organic matter
362 (DOM) of water, which is known to contain as major components oxidized sterols (Woods et
363 al., 2012), terpenoids (Lam et al., 2007), and other lipids (Edith Kaiser et al., 2003).

364 3.5 Metabolomic fingerprint of the FW anaerobic digesters

365 The CC1 component resolved from the FW dataset shows a similar (although less
366 extensive) metabolomic profile to that obtained for the GG dataset. That is, this component is
367 rich in amino acids (5 metabolites, one of them acetylated) and amino acid catabolism products
368 (2 metabolites), as well as nucleic base degradation products (2 metabolites). Hence, CC1 is
369 representative of the hydrolytic stage in anaerobic digesters, where macromolecular compounds
370 such as proteins and DNA from the substrate are broken down into simpler molecules. This CC
371 also includes 2 sulfide compounds, which can be related to hydrogen sulfide formation
372 occurring in the degradation processes of organic matter (Ghaly et al., 2010). Specific to FW,
373 cadaverine and histamine were also detected in CC1 (**Table A.3**). These molecules are known
374 as biogenic amines, or nitrogenous compounds mainly formed by the decarboxylation of amino
375 acids (Kuley et al., 2017). Biogenic amines are one of the many compounds resulting from fish
376 spoilage by microbial growth (Ghaly et al., 2010). All highlighted metabolites in CC1 are
377 associated with positive loadings, denoting a decrease of these compounds over time, as seen
378 in the same component for the GG dataset.

379 Finally, CC2 only includes 4 assigned features. From those, one is a secosteroid, two
380 correspond to alkylated glycerol compounds, and the last one is triethanolamine (**Table S2**). In
381 all cases, these metabolites can be regarded as modified lipids, found predominantly in WAS
382 substrate (**Fig3D**). Since CC2 scores do not change significantly over time (**Fig. 2B**), it may
383 indicate that these transformations took place prior to the anaerobic digestion.

384 3.6 Assessment of the metabolic degradability in AcoD

385 In order to assess from a metabolomics point of view whether the blending of WAS with
386 a co-substrate (GG or FW) improves the degradability of the former, we have calculated the
387 metabolic biodegradability rate (MBR) and the Factor of Synergy in Co-digestion (FSC) for
388 every studied mixture. The MBR indicates how much a mixture is degraded referenced to the
389 100% co-substrate mono-digestion. On other hand, the FSC can be used to identify whether or

390 not the MB of the tested co-digestions are equivalent to the ones obtained by digesting the two
391 substrates separately. More information about these terms is given in section 2.7.

392 The metabolic biodegradability rate (MBR) of GG mono-digestion did not improve in
393 a statistically significant amount from day 14 ($89\% \pm 14\%$, **Fig4A**) to day 21 ($100\% \pm 9\%$,
394 **Fig4A**). For WAS mono-digestion, the MBR was of $18\% \pm 8\%$ at day 14, and of $20\% \pm 1\%$ at
395 day 21 (**Fig4A**). When the two substrates were used in mixture, the best improvement in the
396 MBR was found for 50% WAS/50% GG AcoD after day 14, with almost 11% of observed
397 synergy in the degradation ($FSC = 1.11$, **Fig4C**). 25% WAS/75% GG AcoD also showed a
398 synergy effect in the degradability ($FSC = 1.08$, **Fig4C**). However, at day 21, all tested mixtures
399 presented a FSC below 1, indicating that the degradation pathways triggered by mixing together
400 the two substrates are less efficient than those employed during WAS and GG mono-digestions
401 (**Fig4C**).

402 On another hand, in terms of MBR, neither the WAS mono-digestion nor the FW mono-
403 digestion were improved in a statistically significant proportion between day 21 and day 28
404 (from $31\% \pm 1\%$ to $24\% \pm 15\%$, and from $103\% \pm 3\%$ to $100\% \pm 2\%$, respectively (**Fig4B**)). This
405 can be interpreted as the degradation of organic molecules being completed before day 21, as
406 observed in the heatmap representation of **Fig3**. When the two substrates were mixed and
407 digested, a higher MBR than the $MBR_{theoretical}$ was obtained for all tested conditions (**Fig4B**).
408 In graphical terms, in **Fig. 2B**, it can be observed that scores from 25% (orange), 50% (brown)
409 and 75% FW (purple) at day 0 are located outside of the theoretical triangle that describes the
410 metabolic variability of this AcoD system, causing that the calculated MB are larger in those
411 conditions than in 100% FW mono-digestion (blue). Our hypothesis is that the mixing of the
412 two co-substrates produced an additional breakdown of WAS macromolecules before the start
413 of the digestion process. As a consequence, at the initial time-point, samples from co-digestion
414 experiments were found to be metabolically more concentrated than 100% FW samples (shown

415 in **Fig3B**). Due to this additional process, the best AcoD was found for 75% WAS/25% FW
416 AcoD after 28 days with an increase of more than 100% in the MBR from the theoretical value
417 (FSC = 2.13, **Fig4D**). 50% WAS/50% FW and 25% WAS/75% FW AcoD also showed an
418 improved MBR (FSC of 1.58 and 1.43, respectively (**Fig4D**)). The synergy effect observed in
419 these 3 mixtures after 28 days was slightly higher than that after 21 days (**Fig4D**).

420 In summary, we demonstrated that metabolomics can be used to assess the anaerobic
421 co-digestion of sludge with a co-substrate using two different AcoD systems. This methodology
422 can be therefore applied to investigate the improvement in the co-digestion associated to other
423 co-substrates. Finally, it must be noted that this methodology is potentially easy to extrapolate
424 to the waste management industry, since it is not restricted to only HPLC-MS data, and any
425 metabolomics-based high-throughput technique, including NMR and GC-MS, can be used for
426 the same purpose.

427 **4. Conclusion**

428 A chemometrics-based metabolomics strategy to determine the metabolic fingerprint of
429 AcoD and to assess substrate degradability during AcoD was developed.

430 On one hand, regarding the two AcoD studied systems, a comprehensive insight of the
431 metabolic processes occurring was achieved. We observed that most of the metabolic
432 alterations over time that occurred during AcoD were linked to the co-substrate, the compounds
433 in the co-substrate being more biodegradable than those in WAS. For GG AcoD, these
434 alterations were mainly linked to protein degradation processes. For FW AcoD, the two most
435 important groups of compounds being biodegraded over time were the amino acids and the
436 biogenic amines.

437 On the other hand, regarding the assessment of AcoD, the addition of green waste in the
438 AD did not improve the MBR of wastewater sludge, whereas a synergistic effect in the MBR

439 was observed when fish waste was used. The most improved MB in fish co-digestion was found
440 when WAS was mixed with 25% FW.

441

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445

446 **6. Bibliography**

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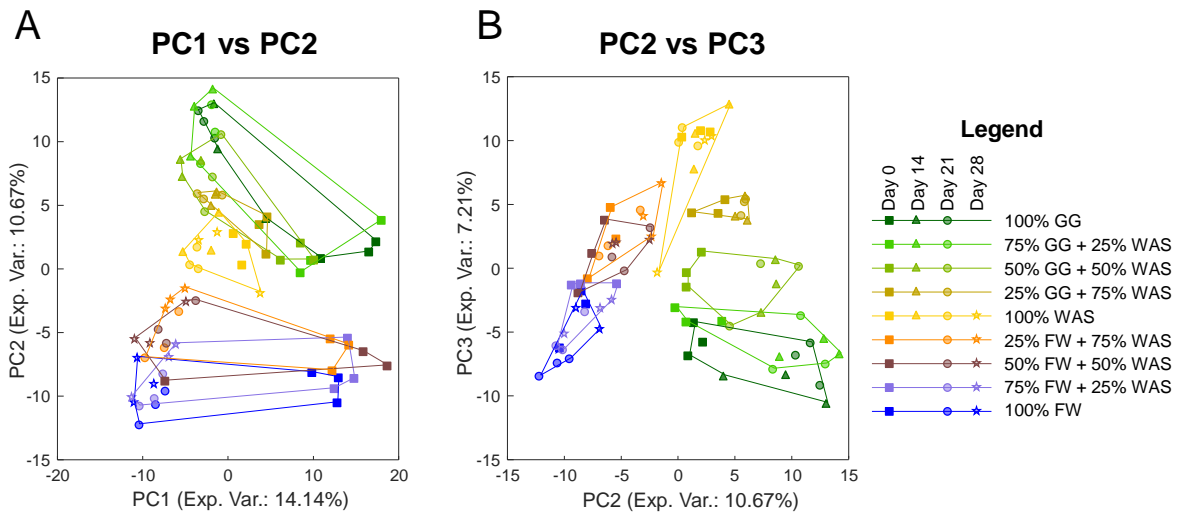
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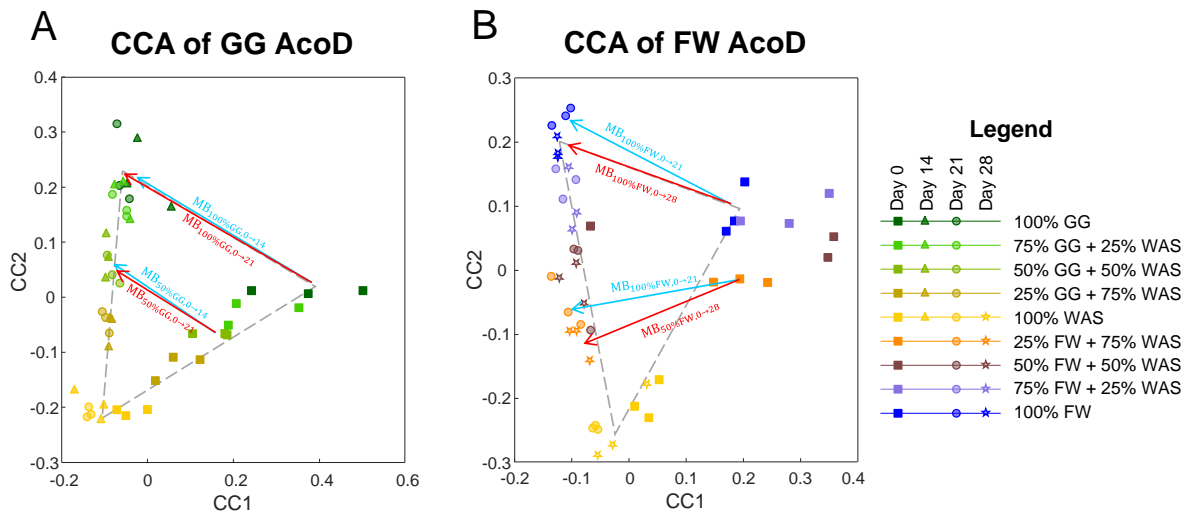
589 **Figures:**



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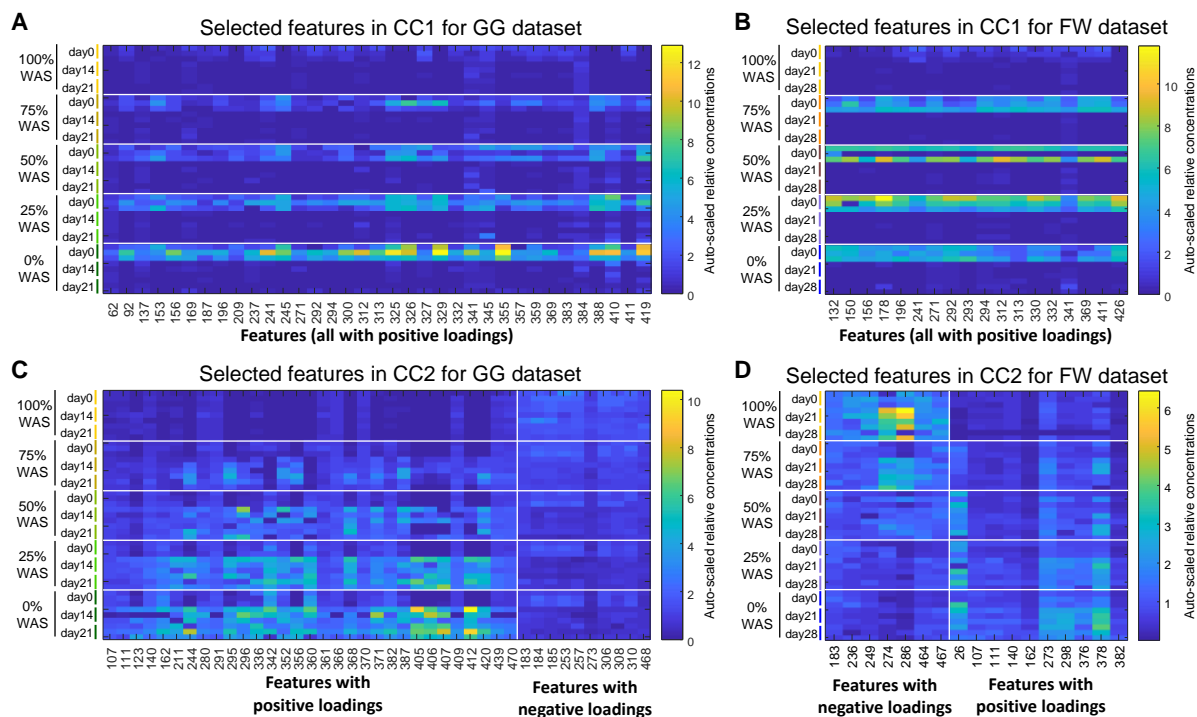
591 **Fig. 1.** Principal Components Analysis: **A.** Scores plot of PC1 vs PC2. **B.** Scores plot of PC2 vs PC3.

592 The substrate composition is given by the color and the sample collection time is represented with
 593 different symbols.



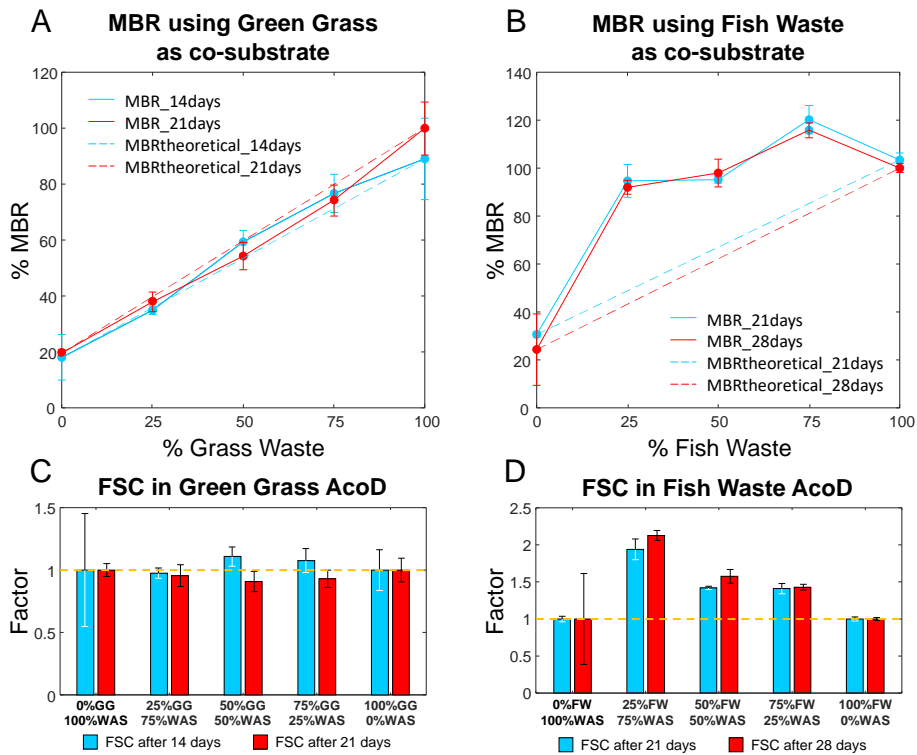
594

595 **Fig. 2.** CCA scores. **A)** CC1 vs CC2 scores on GG dataset. **B)** CC1 vs CC2 scores on FW dataset.. In
 596 both plots, scores distribution shape is similar to a triangle (drawn using dashed grey lines). In **A** and **B**,
 597 the metabolic biodegradabilities (MB) of 50% GG and 100% GG (**A**), and 50% and 100% FW (**B**) linked
 598 to the anaerobic digestion at the two screened time-points days are illustrated with arrows. Cyan arrows
 599 denote the MB achieved at the second time-point, while red arrows denote the corresponding MB at
 600 the third time-point.



601
 602 **Figure 3.** Heatmap representation of temporal evolution of the relative concentration for the selected
 603 features. **A)** Selected features in CC1 for GG dataset. **B)** Selected features in CC1 for FW dataset. **C)**
 604 Selected features in CC2 for GG dataset. **D)** Selected features in CC2 for FW dataset. Putative metabolite
 605 names for the shown features can be consulted in **Table A.2** and **Table A.3**.

606



607

608 **Fig4.** Metabolic biodegradability performance. **A-B)** Metabolic Biodegradability Rate (MBR) for the
 609 different conditions screened. A %MBR of 100 corresponds to the anaerobic digestion of only co-
 610 substrate during the longest period (21 days for GG (**A**), 28 days for FW (**B**)). **C-D)** Factor of Synergy
 611 in CoD (FSC) for the studied mixtures and the two CoD durations.