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1 Long term alkaline storage and pretreatment
2 process of cover crops for anaerobic digestion

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7 Abstract

8 The aim of this work was to study an innovative alkaline process on two cover crops. CaO load of
9 60g.kgTS^{-1} was implemented to combine the functions of storage and pretreatment. Lab-scale
10 reactors were monitored for 180 days to assess the effect of this process on the physico-chemical
11 properties of the biomass. From the first days, pH was not maintained in an alkaline zone and
12 microbial fermentation activity was observed with the degradation of available carbohydrates and
13 production of metabolites, CO_2 and H_2 . High butyric acid accumulation was observed and mass
14 losses of 18.1% and 9.0% of initial VS occurred for oat and rye, respectively. However, no methane
15 potential loss was recorded in the short and long term and the crops were efficiently preserved.
16 The pretreatment had no major impact on fiber solubilization, and no increase in BMP was
17 obtained, which was attributed to the short duration of the alkaline conditions.

18 Keywords

19 Biomethane; Biogas; Catch crops Storage; Pretreatment; Silage fermentation

20 1 Introduction

21 The production of biomethane by anaerobic digestion (AD) is likely to increase greatly in the
22 coming years, as it is expected to replace part of the natural fossil gas. A large variety of organic
23 wastes can be valorized such as sewage sludge, biowastes, manure and crop residues.
24 Additionally, in order to supply a sufficient biomass to meet the demand for biogas, crops are also
25 grown to feed agricultural biogas plants. These crops are interesting substrates for AD since they
26 have a high CH_4 yield and allow a secure feed stock for the biogas plants. However, the cultivation
27 of energy crops is in competition for arable land with food crops, which questions their

28 sustainability as a substrate for AD (Jury et al., 2010). Therefore, cover crops (CC), also called catch
29 crops, are receiving an increasing interest as an alternative to energy crops. These crops are not
30 being grown in place of food/feed crops, but during the intercropping period of their crop rotations.
31 Thus, they avoid the bare soil period that may happen between two food crops cultivation. The
32 use of cover crops in farming practices has both agricultural and environmental benefits, since it
33 allows to avoid erosion and nutrient leaching (Igos et al., 2016; Jian et al., 2020; Sapkota et al.,
34 2012), improves the accumulation of organic matter in the soil (Jian et al., 2020) and facilitates the
35 control of undesirable weeds (Büchi et al., 2020). However, the use of CC for biogas production
36 still faces some difficulties. First, the harvest only takes place once or twice a year, while the
37 digesters are fed continuously. CC are often grown during short and unfavorable periods, and
38 consequently their biomass yields are variable and unpredictable from one year to another. These
39 two aspects make an efficient storage of the harvested crops a mandatory key point in their use
40 for AD.

41 Currently, the storage step is performed by ensiling, a process widely used for forage storage for
42 cattle feeding since the end of the 19th century (Goffart, 1877) . Ensiling relies on the spontaneous
43 fermentation of the substrate under anaerobic conditions, allowing the release of organic acids
44 (mostly lactic acid) and acidification of the medium (Elferink et al., 1999; Weinberg and Ashbell,
45 2003). The low pH under anaerobic conditions inhibits the microbial activity, thus ensuring the
46 substrate stability over several months (Driehuis et al., 2003). When executed properly, ensiling
47 allows a long-term preservation of the biomass energy potential, but various degradations may
48 occur, depending on a large panel of parameters from the crop characteristics to the silo
49 conception (Teixeira Franco et al., 2016). If this process has been optimized for animal feeding for

50 decades, its application to anaerobic digestion is still relatively new and may be improved. New
51 storing methods may also be developed, specifically designed for anaerobic digestion.

52 Another limit in the use of CC for AD is related to their physicochemical characteristics. The cell
53 walls are composed of a complex lignocellulosic matrix made of cellulose, hemicellulose and lignin.
54 This structure limits the access of the microorganisms to the highly degradable carbohydrates of
55 the cellulose and hemicellulose, lowering the yield and the kinetic of the crop conversion into
56 methane (Monlau et al., 2013b). In order to enhance the anaerobic digestion of such
57 lignocellulosic matrix, many types of pretreatment processes have been reported in the literature
58 and their actions on lignocellulose have been well described. Among the different technologies,
59 alkaline pretreatments are particularly relevant since alkaline conditions are highly effective in
60 solubilizing lignin (Carrere et al., 2016). High increases in biochemical methane potential (BMP)
61 were obtained by applying intensive conditions of pretreatments such as high temperature
62 (Sambusiti et al., 2012) or high chemical concentration (Jiang et al., 2017). In addition, significant
63 improvements in BMP were also observed in mild conditions, using NaOH, CaO or KOH, allowing
64 much lower energy consumption (Khor et al., 2015; Thomas et al., 2018). In these conditions, the
65 extension of the duration had a positive effect on the pretreatment effectiveness, and the
66 treatment duration appeared, under certain conditions, to be more favorable than the reactive
67 concentration (Thomas et al., 2018). Despite its lower efficiency when compared to other alkaline
68 compounds like NaOH and KOH, lime presents the advantage to be less expensive. Additionally,
69 CaO is already used on agricultural sites for soil liming to control acidity. Consequently, on an
70 agricultural biogas plant, the use of lime is much more compatible with the use of digestate as a
71 fertilizer than NaOH that causes an increase in the soil salinity due to sodium.

72 If alkaline pretreatments may constitute an interesting option for lignocellulosic biomass such as
73 cover crops, applying them on stored silage does not constitute an adequate solution because of
74 their high concentrations in acidic compounds such as lactic and acetic acids. A large amount of
75 alkaline chemical would be necessary to neutralize the acids and increase the pH. For this reason,
76 the implement of the alkaline pretreatment just after harvesting and extension of the
77 pretreatment reaction time up to several months may constitute an alternative to ensiling for the
78 storage of cover crops, while maximizing the alkaline treatment action. Compared to silage, which
79 leads to a drop in pH in acidic regions (pH=4), microbial inhibition, which is necessary to maintain
80 the methanogenic potential of the substrate, can be ensured by maintaining alkaline conditions.

81 However, as the addition of an alkaline agent is currently mostly used for pre-treatment process
82 purposes and not for storage purposes, the duration of the treatment reported in the bibliography
83 usually ranges from a few hours to 1 or 2 days and very few studies investigated longer duration of
84 up to 7 days (Atelge et al., 2020). Digman et al. (2010) reported an alkaline pretreatment +
85 anaerobic storage process of switchgrass and reed canarygrass using CaO for ethanol production.
86 Several CaO loadings from 14.6 to 100g.kg⁻¹ were applied with storage durations ranging from 30
87 to 180 days. The authors reported a good preservation of the substrate in all cases, even if no
88 global energy balance including mass losses was calculated. In this study, the pretreatment
89 efficiency increased together with lime loading and the highest ethanol yields were obtained with
90 85 and 100g.kg⁻¹ CaO. However, the two studied solid content were very high (43 and 66%TS) and
91 it was observed that moisture content had a positive effect on the pretreatment efficiency. This
92 may suggest that interesting results with lower CaO loading can be obtained in wetter conditions.
93 Finally, even if a correlation between the added CaO amount and the final pH of the silos was
94 reported, no signs of undesirable fermentations were stressed. However, no dynamic monitoring

95 of the variation of the physico-chemical characteristics was made, making difficult the
96 interpretation of the possible metabolic pathways that took place and fine understanding of the
97 process.

98 The aim of this study is to evaluate the mechanisms involved during a long-term alkaline storage
99 (i.e., CaO) of two cover crops, and its impact on the conservation/improvement of the energy
100 potential in anaerobic digestion. The operating conditions were chosen to limit the pretreatment
101 cost, following a procedure similar to ensiling, except the CaO addition as a dry powder before
102 closing the silo. The conditions were set at ambient temperature. The main novelty of this work is
103 the high solid content of the alkaline pretreatment, and the extended reaction duration until
104 fulfilling the storage function. The dynamical changes in the physico-chemical characteristics of the
105 substrate were observed over a six-month period as well as the impact on the methane potential.

106 2 Materials and Methods

107 2.1 Feedstock

108 Two cover crops were used for this experiment. An oat sample (*Avena sativa*) was collected on an
109 experimental agricultural site (Arvalis, Montardon 64121, France) and a rye sample (*Secale*
110 *cereale*) on another agricultural site (Biométharn, Aiguefonde 81200, France). Both crops were
111 grown as winter cover crops, sowed in mid-October and harvested in the very last days of April at
112 maturity stage BBCH 60 (beginning of flowering) and BBCH 59 (end of heading) for oat and rye,
113 respectively. The crops were hand-harvested at approximate cutting height of 10 cm and stored
114 overnight in sealed plastic bags as whole plants at 4°C before use.

115 2.2 Alkaline storage conditions

116 Prior to the experiment, the crops were chopped into 1-2 cm pieces using a garden shredder (AXT
117 2550TC, Bosch GmbH). Then, the alkaline reagent CaO was added as a dry powder and was
118 homogenized manually. A CaO loading of $60\text{g.kg}_{\text{TS}}^{-1}$ was chosen as a compromise between
119 pretreatment efficiency and reactive cost according to the literature. Jiang et al. (2017) found the
120 most interesting lime load for $70\text{g.kg}_{\text{TS}}^{-1}$ $\text{Ca}(\text{OH})_2$ (equivalent to $53\text{g.kg}_{\text{TS}}^{-1}$ CaO) while Thomas et al.
121 (2018) and Khor et al. (2015) obtained significant BMP improvement for $50\text{g.kg}_{\text{TS}}^{-1}$ CaO and 75
122 $\text{g.kg}_{\text{TS}}^{-1}$ $\text{Ca}(\text{OH})_2$ (equivalent to $57\text{g.kg}_{\text{TS}}^{-1}$ CaO) respectively. The storage experiments were
123 conducted in 2.6 L glass flasks sealed with air-tight lids equipped with a rubber septum that allows
124 gas sampling, pressure measurement and pressure release. The experimental setup was inspired
125 by the one used by Hillion (2017), which provided a high repeatability. For each crop, 5 replicates
126 were prepared to be sacrificed after 2, 7, 21, 60 and 180 days to monitor the impact of the
127 pretreatment on biomass conservation and the variation of the physico-chemical properties. 700 g
128 of samples were introduced in each flask just after mixing with CaO, and packed manually until a
129 density of 500gFM.L^{-1} . The flasks were finally flushed with N_2 , sealed and stored in a dark place at
130 22°C until their opening.

131 2.3 Silo monitoring and sampling

132 Gas production and mass losses were monitored regularly all over the experiment. The volumetric
133 gas production was measured by a pressure difference method. Gas sampling and pressure release
134 were made as often as necessary depending on the gas production kinetics. The pressure was
135 measured through the septum with a manometer (Keller LEO 2). Gas was released when pressure
136 exceeded 1.2 bar, and the flask was weighted. The volume of gas inside the flasks (headspace +

137 pore space) was calculated by subtracting the volume of added substrate to the flask volume. The
138 volume of the substrate was calculated using the theoretical density equation adapted from
139 McNulty *et al.* (1982) (Eq.(1)):

$$140 \quad \rho_t = (1 - C) [(M/\rho_l) + (1 - M)/\rho_s] + C/\rho_{CaO} \quad (1)$$

141 where M = fractional moisture content related to fresh matter (FM); C = CaO content related to
142 fresh matter; ρ_l = water density = 1000 kg.m⁻³; ρ_s = dry matter density = 1421 kg.m⁻³ and ρ_{CaO} = CaO
143 density = 3345 kg.m⁻³. The gas composition was analyzed using a gas chromatography (Perkin
144 Elmer Clarus 580) as described in section 2.4.

145 For each sampling date, one flask was opened after measuring final gas volume and composition
146 and weight. The whole sample was homogenized and mixed using a knife mill (Pulverisette 11,
147 Fritsch). Samples were prepared and stored for BMP test by freezing around 2 g_{TS} of matter at -
148 20°C. Total solids (TS) and volatile solids (VS) were measured directly on the samples in triplicate.
149 Water extraction was performed for the measurement of pH, water-soluble carbohydrates (WSC),
150 volatile fatty acids (VFA) and other metabolites. 30 gFM of sample were steeped in 150 mL of
151 deionized water for 18 h at 4°C in sealed plastic pots in triplicate, as suggested by Porter & Murray
152 (2001). Each liquid extract was used separately for pH and soluble compounds analysis. pH was
153 measured directly after extraction on the mixture. Then, the liquid phase was separated by
154 centrifugation (18750 g, 20 min, 4°C) and frozen in air-tightly closed tubes for further WSC, VFA
155 and metabolites analysis.

156 2.4 Physicochemical analysis

157 TS were measured in triplicate by drying 30 g of sample at 105°C for 24 h. Volatile Solids (VS) were
158 then measured by calcination of the dry residue (550°C, 3 h). Pretreated substrates can contain

159 volatile fatty acids (VFA), lactic acid (LA) and some alcohols that may evaporate during oven
160 drying, causing underestimation of TS and VS. For this reason, the TS content value measured by
161 oven drying was corrected using the equation proposed by Porter & Murray (2001) (Eq. (2)):

$$162 \quad TS_C = TS_M + 0.375 LA + 0.892 VFA + 0.975 Alcohols \quad (2)$$

163 where TS_C = corrected TS; TS_M = measured TS; LA = lactic acid concentration; VFA = total VFA
164 concentration; Alcohols = total alcohols concentration. All concentrations are in g.gFM^{-1} .

165 pH was measured in triplicate directly on the extraction mixture using a WTW® SenTix® 41 probe
166 on a WTW® inoLab® pH7110. WSC and concentration of metabolites (i.e., lactic acid (LA), acetic
167 acid (AA), propionic acid (PA), butyric acid (BA) and ethanol (EtOH)) were measured from the
168 centrifuged liquid phase after filtering (0,2 μm nylon filter) by High Performance Liquid
169 Chromatography on Aminex 4PX-87H column (Bio-Rad) at 45°C. Sulfuric acid (0,005 M; 0,3 mL.min^{-1})
170 ¹) was used as mobile phase. WSC content was calculated as the sum of glucose, fructose, xylose
171 and arabinose. Ammonia concentration was measured on liquid extract by titration with boric acid
172 using a Gerhardt® Vapodest 50s® carousel. The gas composition was analyzed by gas
173 chromatography (Perkin Elmer Clarus® 580). The volume of the gas sample was 200 μL and argon
174 was used as carrier gas (350 kPa at 34 mL min^{-1}). After injection, CO_2 was separated from other gas
175 by a capillary R-Q-bond column (30 m x 0.32 mm). H_2 , O_2 , N_2 and CH_4 were separated on a Rt-
176 Molsieve 5 Å capillary column (30 m x 0.32 mm). Injector and thermal conductivity temperatures
177 were set at 250 °C and 150 °C, respectively.

178 Fiber distribution was analyzed in triplicate using the Van Soest and Wine method (1967) from
179 freeze-dried samples crushed to 1mm theoretical particle size. Water extract (W.EX), neutral
180 detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL) and calcination

181 residue (CAL) content were determined. Water-soluble compounds (W.SOLU), neutral detergent
182 soluble compounds (SOLU), hemicellulose (HEMI), cellulose (CELL) and lignin content (LIGN) were
183 calculated as follow : $W.SOLU = 1 - W.EX$; $SOLU = WEX - NDF$; $HEMI = NDF - ADF$; $CELL = ADF -$
184 ADL ; $LIGN = ADL - CAL$. One-way analysis of variance (ANOVA) was used to test the difference in
185 mean value between the different fiber fractions.

186 The total carbon (TC) and total nitrogen (TN) were determined via an elemental analyzer
187 (FlashSmart®, Thermo Fisher Scientific®) on finely grounded freeze dried samples. TC and TN
188 analysis were not replicated.

189

190 2.5 Biochemical Methane Potential Test

191 BMP was measured in batch assay. Samples were digested in 550 mL flasks with a working volume
192 of 400 mL. Each flask is filled with 5 gTS.L⁻¹ of substrate, 5 gVS of anaerobic inoculum (UASB
193 granular sludge), NaHCO₃ buffer (50g.L⁻¹), and macro- and microelement solutions as described by
194 Monlau (Monlau et al., 2012). Before being closed, the flasks were flushed with N₂ to obtain
195 anaerobic conditions. Triplicate bottles were incubated at 35°C. Controls containing only inoculum,
196 buffer and macro- and microelement solutions were prepared in order to subtract the
197 endogenous methane production of the inoculum from the one due to the samples digestion. The
198 methane production was measured using an automatic batch test system (AMPTS® II, Bioprocess
199 Control®, Sweden). Methane potentials are expressed as the volume of methane produced per
200 amount of VS added for the BMP test, or per initial amount of VS estimated after taking into
201 account the mass losses. BMP expressed in NmLCH₄.gVS_{added} is useful to estimate substrate
202 degradability after storage, while BMP expressed in NmLCH₄.gVS_{initial} is mandatory to evaluate the

203 global balance of the alkaline pretreatment process. One-way analysis of variance (ANOVA) was
204 used to test the difference in mean value between BMP of fresh and stored samples.

205 2.6 Theoretical gas production calculation

206 In addition to the measured volume by pressure difference and gas chromatography, the
207 cumulated gas production at 180 days of CO₂ and H₂ was estimated from the associated
208 metabolites. Since most of the microbial fermentations are accompanied by CO₂ and H₂
209 production, their respective stoichiometric reactions were used in the following equations,
210 adapted from Hillion et al. (2018):

211

$$\begin{aligned} 212 \text{ Cumulated CO}_2 = & \text{ Acetic acid + Ethanol + Propionic acid} \\ 213 & + 2 \text{ Butyric acid + Isobutyric acid} \\ 214 & + \text{ Valeric acid + Isovaleric acid + Caproic acid} \quad (3) \end{aligned}$$

$$\begin{aligned} 215 \text{ Cumulated H}_2 = & 2 \times [\text{Acetic acid + Butyric acid + Isobutyric acid} \\ 216 & + \text{ Valeric acid + Isovaleric acid}] \quad (4) \end{aligned}$$

217 where cumulated CO₂ and H₂ are expressed in mmol.kgVS_{init}⁻¹ and all metabolites concentrations
218 are expressed in mmol.kgVS_{add}⁻¹.

219

220 3 Results and Discussion

221 3.1 Raw material characterization

222 The main characteristics of the oat and rye fresh samples are summarized in Table 1. Both crops
223 show a relatively low TS content when compared to other forage crops for silage making, whose
224 optimal TS at harvest is around 30% (Teixeira Franco et al., 2016). These values are representative
225 of what is expected for cover crops. Molinuevo-Salces et al. (2013) reported an average TS value of
226 $17 \pm 5 \%$ and a variation from 10 to 29%TS for 20 different experimental cover crop assays. Cover
227 crops usually have a low TS at harvest since they are cut before the optimal growth stage.
228 However, since no water was added for the alkaline storage process, the TS value is high when
229 compared to other alkaline pretreatment conditions in the literature, where TS is usually less than
230 or equal to 10% (Khor et al., 2015; Monlau et al., 2013a, 2012; Thomas et al., 2018).

231 Both crops had a low nitrogen content, with C/N ratios of 59 and 38 for oat and rye, respectively.
232 Cereals are reported to have a lower nitrogen content than legumes or grasses in the literature
233 (Kaiser and Piltz, 2004).

234 Water-soluble carbohydrates (WSC) were mostly composed of fructose and glucose, which are the
235 principal primary soluble sugars found in temperate forage grass (Downing et al., 2008). Initial
236 concentrations in WSC of 131 g.kgVS^{-1} and 66 g.kgVS^{-1} were measured for oat and rye, respectively.
237 These are considered as medium (oat) to low (rye) when compared to other crops forages in
238 temperate climate, whose WSC content varies from 50 to more than 300 g.kgVS^{-1} (Kaiser and Piltz,
239 2004). However, if a high WSC amount is essential to ensure a good quality of lactic fermentation
240 in silage making, these soluble sugars can be used as easily accessible and biodegradable
241 substrates for undesired fermentations in the present process. Fermentative metabolites such as

242 VFA, lactic acid or ethanol were not detected in the raw samples, indicating the absence of
243 fermentative activity during harvesting and storage prior to the start-up of the experiments.

244 Both crops present similar composition in fiber constituents in the range of commonly
245 encountered values in cover crops, with relatively high hemicellulose content (Molinuevo-Salces
246 et al., 2013)

247 3.2 Evolution of the chemical characteristics

248 The parameters allowing to follow the possible microbial activity and fermentative pathways
249 involved during storage were monitored all along the experiments. Table 2 presents the variation
250 of pH and ammonia concentration over storage period. Figure 1 shows the profile of the main
251 analyzed components at different studied storage times and the cumulated gas production. Figure
252 2 shows the distribution of the energy potential of the main analyzed compounds (WSC,
253 metabolites and gases) expressed in COD equivalent. Unexpectedly, the pH dropped very quickly
254 after closing the flasks and reached the neutrality in less than one week. At the same time,
255 increasing concentrations of organic acids were recorded. The loss of the alkaline conditions was
256 coupled to a quick WSC fermentation to VFA, lactic acid, CO₂ and H₂ (Figures 1 et 2). From the very
257 first days of the storage, different metabolic pathways occurred one after the other and were
258 associated with the accumulation and/or consumption of different kinds of metabolites (Figure 1).
259 Depending on the type of crop, even if some differences in the profile of metabolite
260 concentrations were observed, four main phases can be identified.

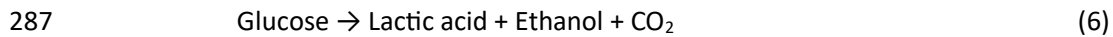
261 During the first two days, a fast and strong accumulation of acetic acid was measured, up to
262 concentration as high as 20.7 g.kgVS_{add}⁻¹ and 25.8 g.kgVS_{add}⁻¹ for oat and rye, respectively.

263 Interestingly, this production of acetic acid was not accompanied by any significant gas

264 production, and occurred at a high pH (between 12 and around 10). If this pH range is not
265 favorable to most of fermentative bacteria, examples of the literature reported high acetic acid
266 fermentation rates at elevated pH of 10 in mesophilic conditions (Dai et al., 2018; Ma et al., 2017)
267 and even 11 at 22°C (Yuan et al., 2006). It was shown that most bacteria could not survive to the
268 alkaline environment (Ma et al., 2017) and the cited works used inoculum from waste water
269 treatment plants or anaerobic reactors with a high initial microbial diversity, which was not the
270 case in the present work. A well-known abiotic chemical reaction, the deacetylation of
271 hemicellulose, could thus be implicated in alkaline conditions. Alkaline deacetylation of
272 lignocellulosic biomasses such as rice straw (Castro et al., 2017) and corn stover (Chen et al., 2014)
273 using a dilute NaOH pretreatment resulted in the release of up to 24 g.kgVS⁻¹ acetic acid by
274 solubilizing the acetyl groups from xylans of hemicellulose. This deacetylation reaction was carried
275 out at high temperature (50 to 80°C), with medium to high reagent concentration (20 to 80g.kgTS⁻¹
276 NaOH), with short reaction time of less than two hours, making obvious the absence of
277 microorganism action in the process. The acetic acid release is strongly associated to the fast pH
278 drop during the first two days of the storage.

279 From day 2 to day 7, whatever the substrate, different soluble fermentative metabolites
280 accumulated, and in particular lactic and propionic acids (oat and rye), acetic acid (oat) and
281 ethanol (rye). The evidence of fermentative activities is also confirmed by the degradation of
282 glucose and fructose with a major decrease of their initial concentration. An increase in CO₂ and H₂
283 production rates was observed. The nature of the consumed sugars and produced metabolites,
284 including gases, indicate that heterolactic fermentation occurred, following one or the other of the
285 reactions below (Eq. (5) and (6)) (Hillion et al., 2018):





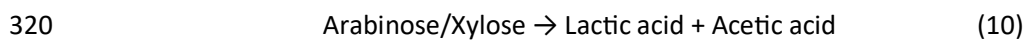
288 Heterolactic fermentations are commonly met in silage fermentation. However, the optimal pH
289 range for the growth of the involved lactic acid bacteria is in the range of 5 to 6, and their growth
290 rate is often reduced if the pH of the crop to be ensiled is equal to or greater than 7 (Driehuis et
291 al., 2003) even if some lactic fermentations were reported with a starting pH of 9.5 using selected
292 *Lactobacillus* strains as inoculum. This may evidence the existence of heterogeneous zones in the
293 substrate with a lower pH than the measured value, allowing the development of lactic acid
294 bacteria. The pH heterogeneity was probably due to the application of CaO as a dry powder
295 and/or the acetic acid release during the deacetylation phase.

296 From the second week of storage until day 60 (oat) or 180 (rye), acetic acid concentrations were
297 increasing, while certain metabolites accumulated after one week of storage were completely
298 (lactic acid, ethanol) or partially (acetic acid, propionic acid) converted into butyric acid. During
299 this period, residual glucose and fructose were completely consumed. High gas production was
300 observed during this phase, and the ratio between CO₂ and H₂ production flow rates remained
301 stable at CO₂/H₂ = 2.5. Butyric fermentation is due to bacteria of the *Clostridium* genus, which
302 commonly grow in insufficiently acidified silages. They grow on soluble carbohydrates (Eq. (7),
303 (Hillion et al., 2018)) and organic acids by a fermentation pathway that can be considered as the
304 sum of Eq. (8) and (9) (Driehuis et al., 2003).



308 In storage processes for anaerobic digestion, clostridial fermentations are considered as
309 undesired, since they lead to the production of H₂ that escapes from the silo and induces losses of
310 methane potential (Kreuger et al., 2011; Teixeira Franco et al., 2016).

311 In the present case, the total consumption of the previously produced lactic acid indicates that, at
312 least, clostridial fermentations of lactic acid occurred. However, regarding the balance between
313 degraded COD from initial WSC and cumulated metabolites (Figure 2), the observed sugar
314 consumption could not explain alone the accumulation level of primary metabolites (LA, AA, PA,
315 EtOH) and their further conversion into BA. This indicates that more complex carbohydrates were
316 constantly degraded and released soluble sugars. These released WSC, mostly xylose, glucose and
317 arabinose from the hemicellulose, may have been continuously converted into lactic acid (Eq. (5)
318 or (10) (Hillion et al., 2018)) and then into butyric acid (Eq. (8) and (9)), or directly degraded into
319 butyric acid following Eq. (7).



321 The clostridial activity may also be evidenced by the fermentation of amino acids and amines into
322 ammonia (Hillion et al., 2018). Table 2 shows a constant increase in ammonia concentration in rye,
323 reaching 22% of initial TN after 180 days of storage. Surprisingly, a much lower ammonia
324 accumulation was observed in oat, despite an important butyric fermentation. This could be
325 explained by the fact that butyric fermentation started earlier in oat, with BA apparition before 7
326 days of storage, when pH was still lowering from 10.5 to 6.6. In these conditions of high pH, a large
327 fraction of the ammonia may have undergone a faster volatilization in the form of NH₃. Total
328 Nitrogen analysis could help to conclude on this hypothesis, but no such measurements were
329 performed on stored samples in the present work.

330 During the last phase of the process, the microbial activity was considerably reduced, except an
331 acetic acid production that continued following a slow kinetic. Oat samples reached this relative
332 steady state 3 times faster than rye samples after only 60 days and the final concentration in BA
333 was twice higher, what may be explained by the higher initial amount of WSC in oat. Interestingly,
334 this state of relative stability began after BA reached a threshold value of $100\text{g}\cdot\text{gVS}_{\text{add}}^{-1}$, after what
335 no further BA production was observed. BA may therefore have an inhibitory effect. However, the
336 fact that rye samples also seem to reach a similar stable state at the end of the experiment from
337 day 108, even with a 2.7 times lower BA amount, indicates that the decrease of the microbial
338 activity is more probably due to the depletion of the accessible fermentation substrates.

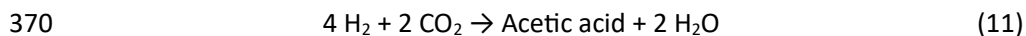
339 3.3 Process impact on Fiber distribution, VS and BMP preservation.

340 The pretreatment effect on the fiber distribution of the crops was studied, and the results are
341 presented in Figure 3. In Oat samples, the three most accessible fractions significantly varied, from
342 39.6 to $31.6\%TS_{\text{init}}$ (W.SOLU), 1.1 to $4.3\%TS_{\text{init}}$ (SOLU) and 26.3 to $11.8\%TS_{\text{init}}$ (HEMI) fractions
343 significantly varied ($p < 0.05$). Rye samples undergone a lower transformation, with significant
344 changes ($p < 0.05$) for SOLU (27.0 to $25.3\%TS_{\text{init}}$) and HEMI (29.4 to $17.7\%TS_{\text{init}}$) fractions only. In
345 both cases, no solubilization of the cellulose and lignin fractions were observed. The hemicellulose
346 was greatly reduced, and easily accessible carbohydrates were released, mostly xylose, glucose
347 and arabinose. This WSC release explains the difference in the balance between the consumed
348 COD of the initial WSC and the accumulated COD of the metabolites (section 3.2 and Figure 2).
349 Nonetheless, since no effect on cellulose and lignin were observed, the pretreatment action of
350 CaO was seemingly limited, and the long-term effect of the pretreatment on lignin wasn't
351 obtained.

352 Figure 4 shows the dynamics of mass losses for both crops over the whole storage period. The gas
353 production related to microbial fermentations was responsible for significant VS losses of 18.1%
354 and 9.0% respectively for oat and rye during the experiment. The measured values were higher
355 (oat) or comparable (rye) to the ones that are commonly observed in ensiling, where 5 to 12% TS
356 losses occur during the fermentation of properly managed silages (Kaiser and Piltz, 2004).

357 The VS losses were mostly due to CO₂ production. Mass losses and cumulated CO₂ production
358 followed the same behavior (Figures 1 et 3). Considering mass balance during alkaline
359 pretreatments, mass losses computed on the base of the mass of gas release were 123 and 40
360 g.kgVS_{init}⁻¹ for oat and rye respectively and were significantly lower than direct mass measurement
361 of the jars (181 and 90 g.kgVS_{init}⁻¹) respectively. This may evidence the presence of gas leaches on
362 the experimental setup over the 6-month period, as direct weighting of jar is a more reliable
363 method. Considering the theoretical gas production based on the accumulated metabolites
364 calculated as presented in section 2.6, cumulated CO₂ and H₂ should theoretically be of 3572 and
365 4341 mmol.kgVS_{init}⁻¹ for oat, 2207 and 3158.4 mmol.kgVS_{init}⁻¹ for rye.

366 The significant difference between these values and the measured ones presented in Figure 1,
367 particularly considering the CO₂/H₂ ratio, may also indicates that in-situ gas consumption like
368 homoacetogenesis took place, converting H₂ and CO₂ into acetic acid (Eq. (11), (Hillion et al.,
369 2018)).



371 This phenomenon occurs in dark fermentation, where in-situ H₂ consumption by homoacetogenic
372 bacteria is reported, particularly at pH close to 7 (Saady, 2013). Consequently, the effective energy
373 losses due to H₂ production were low.

374 BMP tests were carried out on the raw substrates and on substrates stored for 7, 60 and 180 days.
375 Figure 5 summarizes the methane potential expressed in $\text{NmLCH}_4\cdot\text{gVS}_{\text{add}}^{-1}$ and $\text{NmLCH}_4\cdot\text{gVS}_{\text{init}}^{-1}$, as
376 detailed in section 2.5. The raw oat and rye samples BMP were respectively 294 ± 3 and 348 ± 11
377 $\text{NmLCH}_4\cdot\text{gVS}_{\text{init}}^{-1}$. These values are comparable to other BMP of cereal cover crops that commonly
378 vary from 200 to 400 $\text{NmLCH}_4\cdot\text{gVS}^{-1}$ in the literature (Molinuevo-Salces et al., 2013).

379 Whatever the cover crop, the degradability of the substrate increased during the alkaline
380 pretreatment storage by 29% (oat) and 8.5% (rye) after 180 days, suggesting at first glance a
381 significant ($p = 0.015$ and 0.056 , respectively) action of the pretreatment on the lignocellulosic
382 biomass. However, after taking into account the mass losses during the storage, the methane
383 potential only varied by +6% and -1% for oat and rye, respectively, which indicates no significant
384 difference from the BMP of the fresh substrates ($p = 0.355$ and 0.676 , respectively). During the
385 storage period, the production of H_2 can be the cause of an energy loss, and thus of the reduction
386 of the methane potential. This loss was estimated by calculating the COD of H_2 produced (Figure 2)
387 and it represented 5.4 and 2.2 $\text{NmLCH}_4\cdot\text{gVS}^{-1}$ for oat and rye, respectively 1.8 and 0.6% of initial
388 BMP. This theoretical value is very low compared to that of the BMP of the fresh substrate, which
389 may explain why no loss of methane potential was experimentally observed during storage. In
390 well-preserved silages, slightly higher energy losses from 2 to 4% happen during fermentation
391 (Kaiser and Piltz, 2004). Consequently, during the alkaline storage, because of the loss of mass of
392 the substrate in the form of CO_2 and the conversion of the initial soluble carbohydrates (373 NmL
393 $\text{CH}_4\cdot\text{g}^{-1}$) into more energetic compounds such as butyric acid that were released ($636 \text{ NmLCH}_4\cdot\text{g}^{-1}$),
394 the methane potential was concentrated in the remaining dry matter. Figure 5 presents the
395 estimated BMP of the present metabolites in the substrates, in $\text{NmLCH}_4\cdot\text{gVS}_{\text{add}}^{-1}$. The increase of
396 the BMP of the metabolites was strongly linked to the increase of the substrate degradability and

397 mass losses, showing the concentration of the BMP of the crop in the remaining biomass after
398 storage.

399 Thus, negligible BMP losses occurred considering the whole process, and the initial methane
400 potential of the harvest was efficiently preserved, despite microbial reactions considered as highly
401 undesirable in crop storage processes such as butyric fermentation. By extension, this work brings
402 interesting data for understanding the impact of ensiling on crops for biogas production. In fact,
403 the substrate characteristics along with the microbial fermentation pathways that took place after
404 the first days (pH close to neutrality and important butyric fermentation) is highly comparable to a
405 silage fermentation considered as of a very poor quality. The quality of silage for animal feeding
406 fulfills the following criteria: pH < 4.5, lactic acid > 30g.kgTS⁻¹, butyric acid < 10% of total VFA
407 (Bureenok et al., 2016), while the present stored substrate is far from these characteristics,
408 showing that ensiling for biogas production has very different purposes than for animal feed. This
409 suggests that the ideal silage characteristics for anaerobic digestion are not well defined yet, and
410 future researches may address this subject.

411 Considering the initial pretreatment objective of the process, it can be concluded that the alkaline
412 conditions were not maintained long enough to obtain the expected long-term pretreatment and
413 increase the BMP. However, even if a significant increase is not already reached, the elevated pH
414 (pH 7) of the stored crop could lower the detrimental effect of organic acids on the concrete walls
415 of the silo, which is a reported problem with acid silages (pH 4) (Koenig and Dehn, 2016). Research
416 needs to be undertaken in order to reach the optimal conditions of operation by focusing on the
417 initial characteristics like the alkaline reactive nature and load, global TS and the presence of
418 available substrates for microbial fermentation, with the purpose of maintaining the alkaline
419 conditions for a sufficient time in order to increase the pretreatment efficiency.

420 4 Conclusions

421 A succession of abiotic and biotic reactions induced a fast and sharp pH drop from 12 to 7, and
422 thus compromised the long-term action of the alkaline agent. Despite undesirable fermentations
423 and high mass losses, no significant effect on the methane potential was observed during the
424 storage process. Alkaline storage, however, induced an increase in specific biodegradability which
425 is associated with mass loss. This study clearly demonstrates that the methanogenic potential of
426 the two selected cover crops was maintained for more than 6 months. An optimized process under
427 stable alkaline conditions could therefore allow a better pretreatment efficiency.

428

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433 physical-chemical analysis were performed at the Bio2E platform
434 (doi:10.5454/1.557234103446854E12)

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560 Table and Figure Captions

561 Table 1: Physicochemical characteristics of the catch crops

562 Table 2: pH and NH₄-N variation during storage.

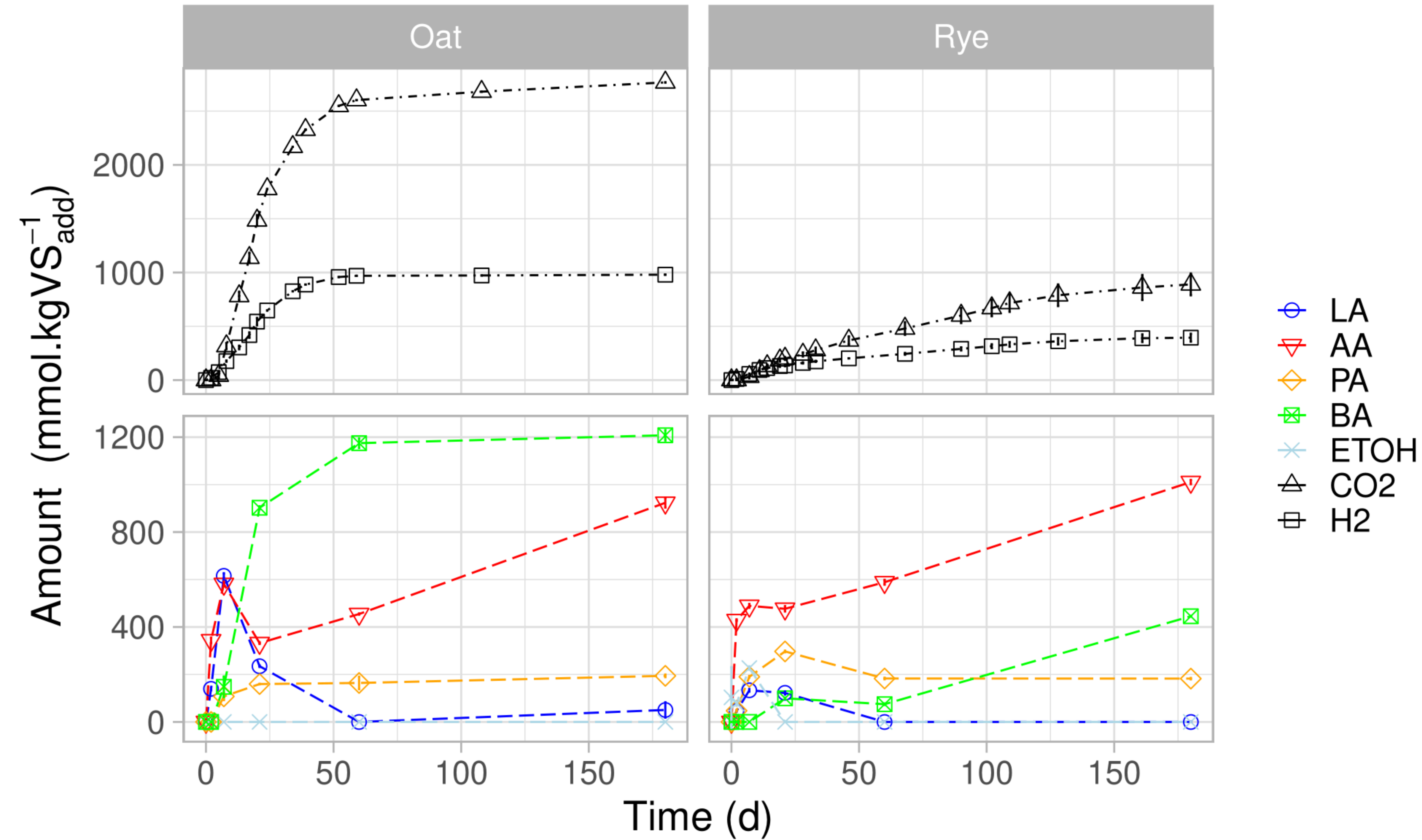
563 Figure 1: WSC, gas and metabolites variation during storage. Lactic acid (LA), Acetic acid (AA),
564 Propionic Acid (PA), Butyric acid (BA) and Ethanol (ETOH) are expressed in mmol.kgVS_{add}⁻¹. CO₂ and
565 H₂ are expressed in cumulated mmol/kgVS_{init}⁻¹. The error bars indicate standard deviation.

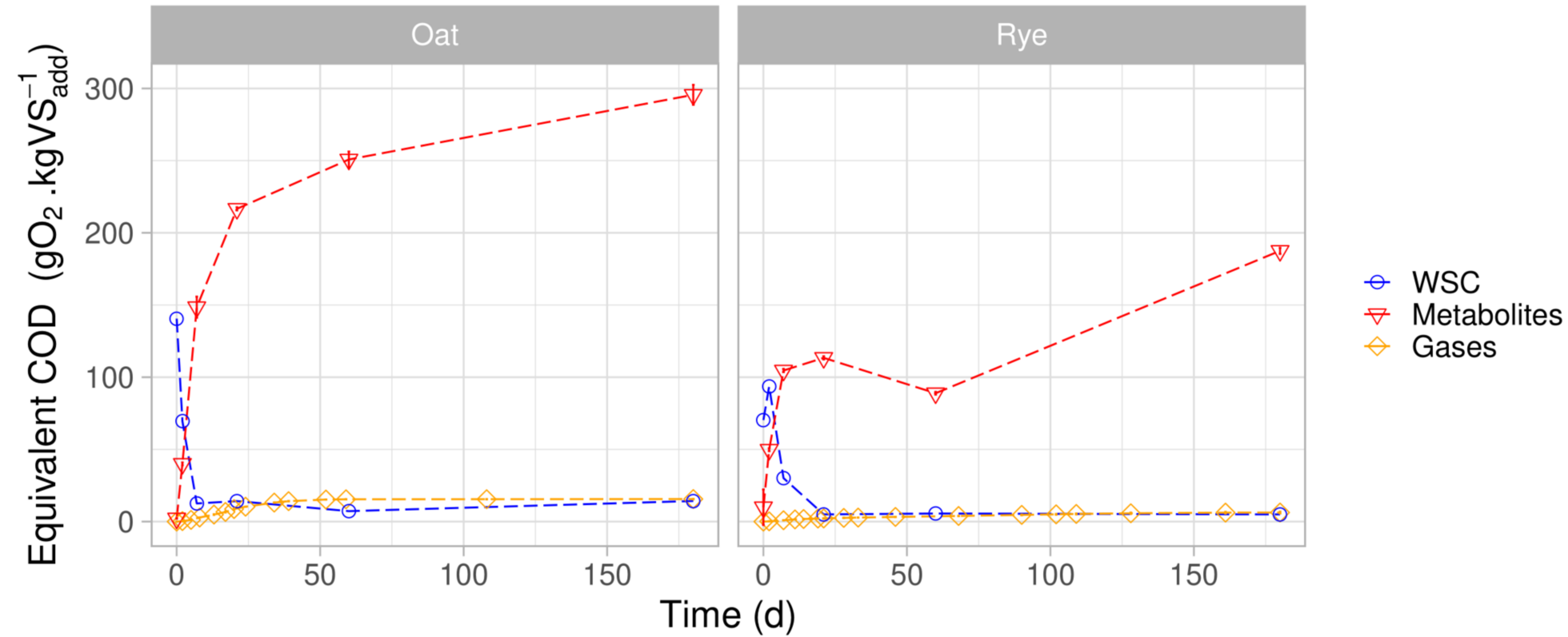
566 Figure 2: Variation of the COD repartition between WSC, soluble metabolites and gases. The error
567 bars indicate standard deviation.

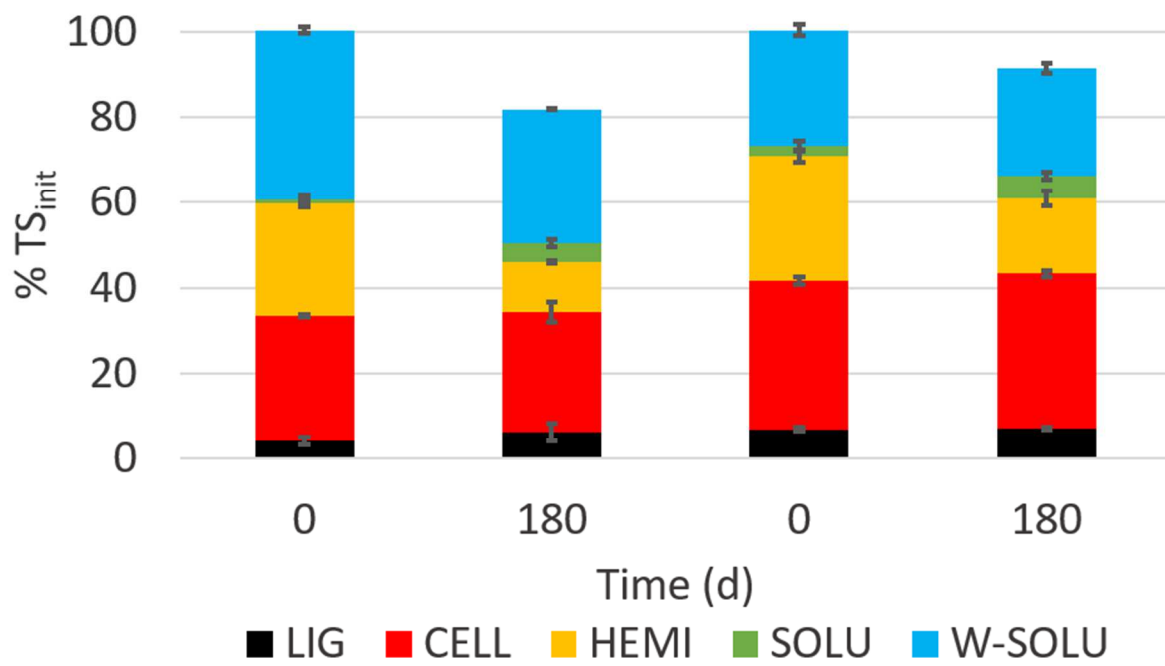
568 Figure 3: Alkaline storage effect on fiber distribution. The error bars indicate standard deviation.

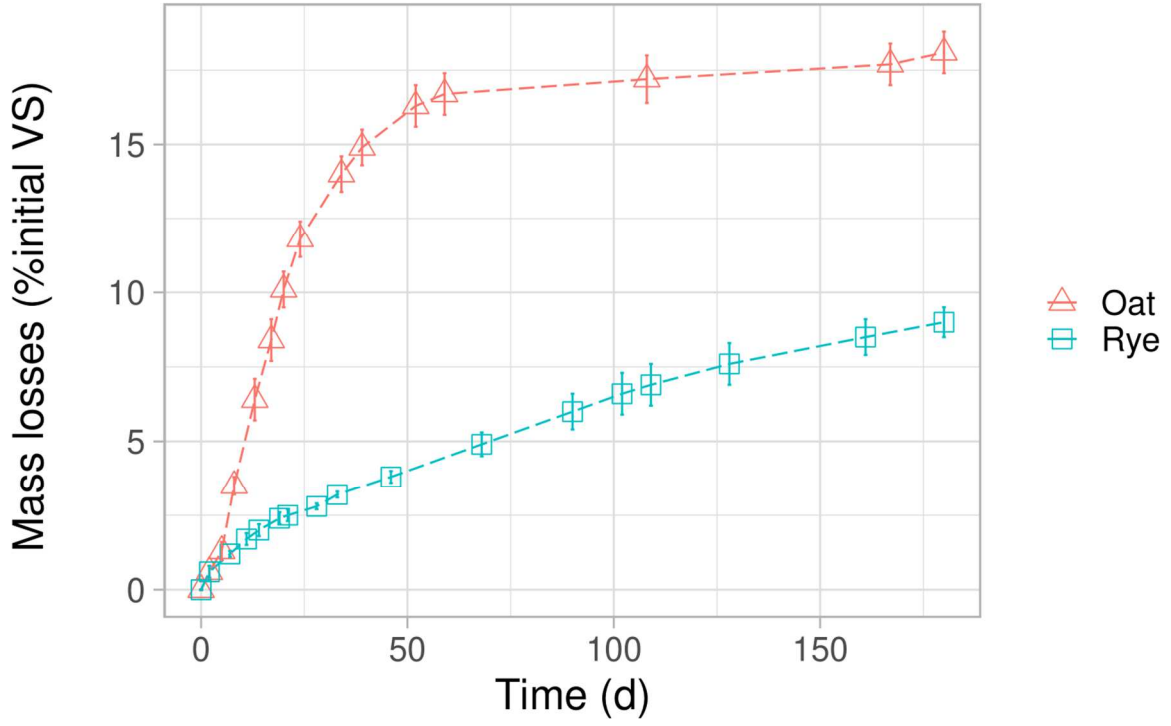
569 Figure 4: Dynamics of mass losses. The error bars indicate standard deviation.

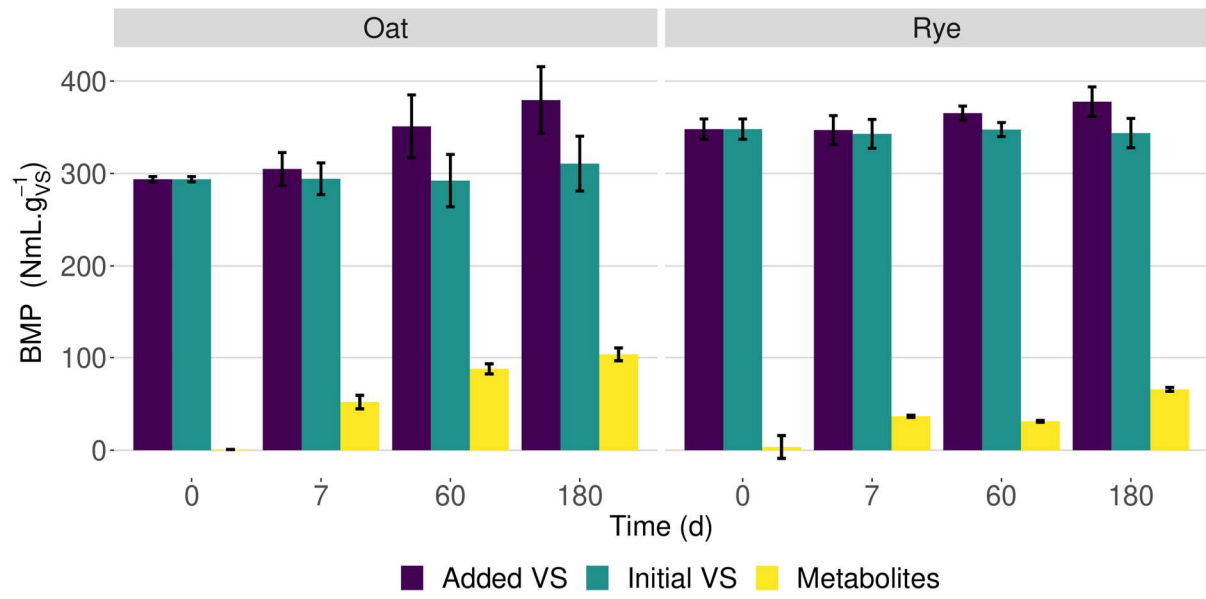
570 Figure 5: Impact of storage duration on methane potential. BMP (NmLCH₄.gVS_{added}⁻¹) and BMP
571 (NmLCH₄.gVS_{initial}⁻¹) represent pretreated crop biodegradability and methane potential reported to
572 the VS amount before pretreatment, respectively. BMP_{metabolites} represent the contribution of
573 soluble metabolites in BMP (NmLCH₄.gVS_{initial}⁻¹). The error bars indicate standard deviation.





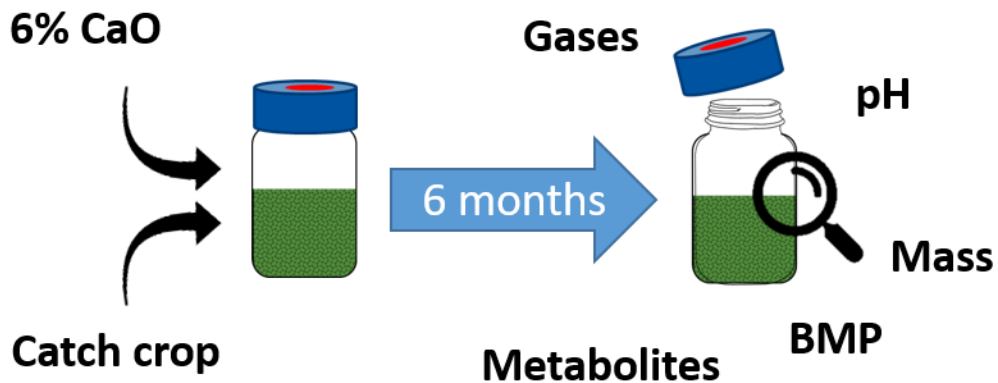
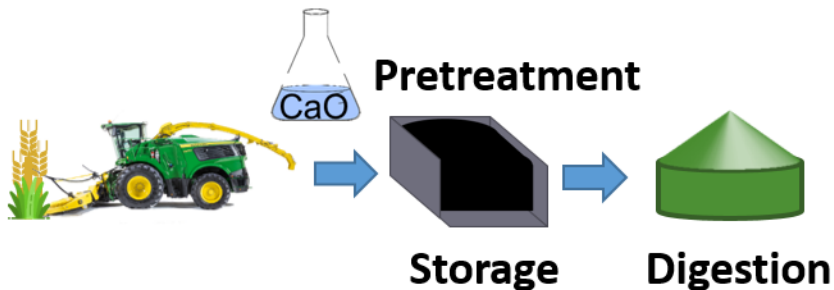






	<i>Oat</i>	<i>Rye</i>
<i>pH</i>	6.0 ± 0.0	6.0 ± 0.0
<i>TS (%FM)</i>	21.8 ± 0.01	24.9 ± 0.03
<i>VS (%FM)</i>	20.8 ± 0.01	23.7 ± 0.03
<i>TN (%VS)</i>	0.7	1.1
<i>TC (%VS)</i>	41.4	42.1
<i>VFA (g.kg_{vs}⁻¹)</i>	< d. l.	< d. l.
<i>Fructose (g.kg_{vs}⁻¹)</i>	76.6 ± 0.8	34.0 ± 0.4
<i>Glucose (g.kg_{vs}⁻¹)</i>	54.7 ± 0.7	31.4 ± 0.1
<i>WSC (g.kg_{vs}⁻¹)</i>	131 ± 1	66 ± 0
<i>EtOH (g.kg_{vs}⁻¹)</i>	< d. l.	< d. l.
<i>W.SOLU (%TS)</i>	39.6 ± 0.7	27 ± 1.4
<i>SOLU (%TS)</i>	1.1 ± 0.8	2.4 ± 1.2
<i>HEMI (%TS)</i>	26.3 ± 0.7	29.4 ± 1.5
<i>CELL (%TS)</i>	29.3 ± 0.2	34.9 ± 0.9
<i>LIG (%TS)</i>	4.1 ± 0.8	6.8 ± 0.5

<i>Time (d)</i>		0	2	7	21	60	180
<i>Oat</i>	<i>pH</i>	11.9 ± 0.1	10.5 ± 0.0	6.6 ± 0.1	7.1 ± 0.1	7.5 ± 0.0	6.9 ± 0.0
	<i>NH4-N (g.kgVS_{add}⁻¹)</i>	0.2 ± 0	0.8 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.3 ± 0.2
<i>Rye</i>	<i>pH</i>	12 ± 0.1	9.8 ± 0.0	7.2 ± 0.0	7.2 ± 0.1	7.9 ± 0.1	7.7 ± 0.1
	<i>NH4-N (g.kgVS_{add}⁻¹)</i>	0.3 ± 0	0.6 ± 0.1	1.1 ± 0	1.7 ± 0.1	2.3 ± 0	2.7 ± 0.1



No alkaline pH stability



Microbial activity



High mass losses



No impact on CH₄ potential