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## Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants

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### ► To cite this version:

Ulysse Bremond, Aude Bertrandias, Denis Loisel, Julie Jimenez, Jean-Philippe Steyer, et al.. Assessment of fungal and thermo-alkaline post-treatments of solid digestate in a recirculation scheme to increase flexibility in feedstocks supply management of biogas plants. *Renewable Energy*, 2020, 149, pp.641-651. 10.1016/j.renene.2019.12.062 . hal-02555213

**HAL Id: hal-02555213**

**<https://hal.inrae.fr/hal-02555213v1>**

Submitted on 21 Jul 2022

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1    **Assessment of fungal and thermo-alkaline post-treatments of solid digestate in**  
2    **a recirculation scheme to increase flexibility in feedstocks supply management**  
3    **of biogas plants**

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10

11    **Abstract**

12    Agricultural biogas plants can suffer occasional feedstock shortages (poor harvest, storage...) and  
13    recirculation of solid digestate (SD) into digester has been identified as a simple way to offset methane  
14    production loss from these situations. Calculations show that recirculation of SD could offset for losses  
15    in plant methane production by up to 2.4%. In that context, two post-treatments were evaluated to  
16    enhance residual potential methane of agricultural SD. Effect of fungal solid state fermentation (SSF)  
17    of SD on subsequent methane production has never been explored before. It was hypothesized that:  
18    (i) ligninolytic fungi would be able to specifically use the complex fraction of SD for their growth and  
19    (ii) energy generation from the subsequent anaerobic digestion of the colonized SD will be enhanced.  
20    However, experiments showed that thermo-alkaline treatment of SD (used as alkalization and  
21    sterilization process) and a high spawn level (20% w/w) were necessary to perform fungal SSF. Besides,  
22    the observed fungal activities on SD did not target specifically the most complex fractions. This led to  
23    uncontrolled organic matter losses and subsequent decreases of biodegradability and methane yield  
24    of SD (up to 50%). Therefore, fungal SSF of SD before its recirculation into biogas plants appeared not  
25    to be a viable option.. Only thermo-alkaline treatment (CaO 2% w/w and 121°C 30 min) enhanced  
26    methane yield of SD by 13% and decreased its complex fraction by 25%. Further studies on  
27    optimization of this post-treatment may enhance efficiency of SD recirculation strategy to offset plant  
28    methane production losses.

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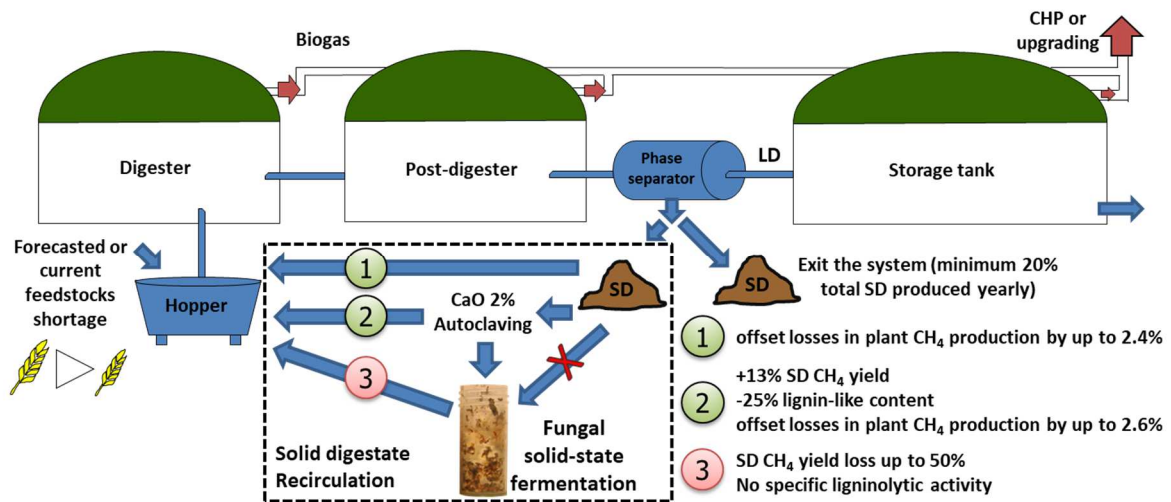
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43 **Graphical Abstract**



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45 **Highlights**

- 46 • SD recirculation could compensate loss in plant methane yield by up to 2.4%.
- 47 • Necessary sterilization and high spawn level to allow fungal SSF of SD.
- 48 • Observation of losses in biodegradability and methane yield of SD after fungal SSF.
- 49 • No specific degradation of the lignin-like fraction of SD during fungal SSF.
- 50 • Thermo-alkaline treatment increases residual methane potential of SD (+13%).

51

52 **Keywords:**

53 Anaerobic digestion  
 54 Solid digestate recirculation  
 55 Fungal post-treatment  
 56 *Pleurotus ostreatus*  
 57 Thermo-alkaline post-treatment  
 58 Agricultural biogas plant

59

60 **Abbreviations**

61 AD, anaerobic digestion; BMP, biochemical methane potential; COD, chemical oxygen demand; CSTR,  
 62 continuous stirred-tank reactor; FM, fresh matter; HRT, hydraulic retention time; LD, liquid digestate;  
 63 NEOM, Non extractable organic matter; OM, organic matter; PEOM, Poorly extractable organic matter;  
 64 PO, *Pleurotus Ostreatus*; REOM, readily extractable organic matter; RMP, residual methane potential;  
 65 SD, Solid digestate; SEOM, slowly extractable organic matter; SPOM, soluble extractable fraction from  
 66 particular extractable organic matter; SRA, *Stropharia rugoso-annulata*; SSF, solid-state fermentation;  
 67 TS, total solids; VFA, volatile fatty acids; VS, volatile solids.

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## 77 1. Introduction

78 The global environmental crisis combined with fossil fuel depletion push governments to gradually  
79 shift their energy mix toward renewable solutions using incentive policies [1]. Among these solutions,  
80 anaerobic digestion (AD) was identified as a key technology as it treats waste to produce energy under  
81 the form of biogas and as it is relatively low cost compared to other existing technologies [2].  
82 Concentrated mainly in Europe, with half of the global biogas production units in 2015, the number of  
83 industrial biogas plants continues to increase and energy production should meet the 2020 EU biogas  
84 energy targets [3]. Agricultural feedstocks are the main biomass deposit in Europe. So, agricultural  
85 biogas plants, mostly based on continuous stirred-tank reactor (CSTR) technology, are most  
86 widespread with 12,500 out of 17,660 existing installations in 2016 [4].

87 This growing industry is searching for innovations in order to improve its biogas production,  
88 conditioning and utilization. Concerning the former point, an optimization of the process is being  
89 sought, notably by a better conversion of organic matter (OM) into biogas via biomass pretreatment  
90 and a finer management of co-digestion [5–7]. Co-digestion, which is commonly practiced in  
91 agricultural plants, can lead to incomplete AD of biomass, especially of lignocellulosic biomass, due to  
92 too short hydraulic retention times (HRT). For instance, to reach 95% of methane recovery from  
93 manure, around 200 days HRT are needed while energy crops only need around 90 days [8].  
94 Incomplete AD due to empirically selected HRT (for instance 100 days) can lead to methane emissions,  
95 in particular from the liquid fraction of digestate storage, hampering environmental benefit [9,10]. One  
96 option to improve methane recovery from biomass is to define good management practices of  
97 digestates from agricultural biogas plants before their land spreading.

98 Concerning the liquid fraction of the digestate (LD), coverage and heating of the storage tank were  
99 identified as a good strategy to enhance both biogas plant yield (longer HRT for liquids) and its  
100 environmental footprint as methane is recovered [11,12]. For the solid fraction of the digestate or solid  
101 digestate (SD), strategies to apply are less obvious. It is generally stored in a composting pile until  
102 stabilization and application as soil amendment. However, it has been proven that SD coming from  
103 agricultural plants can contain residual methane potential (RMP) varying between 24-240 Nm<sup>3</sup> CH<sub>4</sub>·ton<sup>-1</sup>  
104 Volatile Solids (VS) as a function of the feedstocks and applied HRT [13]. SD recirculation into the  
105 plant can be an interesting strategy to enhance plant efficiency by recovering this RMP from SD and  
106 allow a more flexible management of plant feedstocks. Indeed, SD can be recirculated when: (i) there  
107 are some feedstock limitations (in terms of quantity/quality); (ii) feedstocks need to be economized  
108 (forecasted shortage), in that case SD can be integrated to the ration in replacement of feedstocks that  
109 are well stored (no methane loss over time such as well-prepared crop silage). Under these conditions,  
110 additional post-treatment can be performed to further degrade OM of SD (especially lignocellulosic  
111 compounds) and enhance methane production during recirculation. Until now, mechanical,  
112 thermochemical and enzymatic post-treatments have been tested on SD with various results; for  
113 instance, biological post-treatment using an enzyme cocktail on SD succeeded in increasing its  
114 methane potential by 13% [14]. In this recirculation scheme, the use of other types of biological  
115 treatments might be of interest due to their reasonable cost and environmental friendliness [15].

116 Fungal post-treatment of SD has been recently explored to valorize the remaining carbon into value-  
117 added products such as: (i) lignocellulolytic enzymes via submerged fungal fermentation of 21 different

118 strains [16] or via solid-state fermentation (SSF) using *Trichoderma reesei* [17]; (ii) volatile fatty acids  
119 (VFA) via SSF using *Pleurotus Sajor Caju* [18]; (iii) Edible mushrooms since *Pleurotus ostreatus* was  
120 successfully cultivated on up to 60% w/w solid digestate mixed with traditional substrates [19]. But  
121 none of these studies evaluated the impacts of fungal growth on SD methane yield in the case of a  
122 recirculation. Several fungal strains are known to be able to selectively degrade lignin and use it for  
123 their growth during SSF of straw. They can therefore potentially promote the conversion of lignin  
124 concentrated in SD into **fungal biomass that will be** accessible OM for AD microorganisms, which can  
125 subsequently produce methane after recirculation. Two strains were identified as potentially  
126 promising in that view, *Pleurotus Ostreatus* (PO) and *Stropharia rugoso-annulata* (SRA), as they are  
127 both robust, little demanding in cultivating techniques and known to efficiently degrade lignin when  
128 growing on wheat straw [20,21]. Besides, their cultivations are well known at industrial scales.  
129 Compost mushrooms such as *Agaricus bisporus* were not selected as they display lower ligninolytic  
130 activities and are generally more efficient in degrading cellulosic compounds [22].

131 The objective of this study is to evaluate an original process scheme where solid digestate coming from  
132 an agricultural CSTR biogas plant undergoes a post-treatment, which corresponds to a fungal SSF,  
133 before being recirculated back into the biogas plant. Growing conditions for fungal SSF were studied.  
134 The impact of fungi colonization over time on methane yield was analyzed as well as the evolution of  
135 OM composition, especially the lignin-like fraction. Impact of the sterilization, corresponding to a  
136 thermo-alkaline treatment alone on SD was also analyzed. Finally, feasibility at full scale of fungal SSF  
137 in a recirculation scheme in anticipation of a feedstock shortage or to overcome it is discussed.

## 138 **2. Materials and methods**

### 139 **2.1. Solid digestate and fungal mycelium**

140 SD was collected from a French agricultural CSTR plant, directly on-site after digestate mechanical  
141 separation. SD was then stored at -20 °C until the start of experiments in order to avoid any matter  
142 degradation. Biogas plant features are given in **Table 1**. This plant was selected for the following  
143 reasons: (i) a ration that is relatively rich in lignocellulosic biomass; (ii) the use of a screw press, a  
144 covered storage tank and an intermediate HRT which are representative of the process conditions  
145 applied in the agricultural biogas sector [23]. Besides, annual methane production ( $V_{CH_4}$  produced) was  
146 calculated by multiplying the injected biomethane flow rate ( $145 \text{ Nm}^3 \cdot \text{h}^{-1}$ ) by 8,200 hours annual  
147 functioning (taking into account technical maintenance and possible operational contingencies). A  
148 total yearly methane production of  $1,189,000 \text{ Nm}^3$  was obtained. Methane yield ( $84.9 \text{ Nm}^3 \text{ CH}_4 \cdot \text{ton}^{-1}$   
149 feedstock) was obtained by dividing the yearly methane production by the total amount of feedstock  
150 used each year.

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Feedstocks composition	Amount of feedstock (tons/year)	Temperature	HRT (days)	Digestate separation technique	Quantity of SD produced (tons/year)
Catch crop (30%) Bovine manure (18%) Beet pulp (11%) Cereal dust (8%) Whey (35%)	13,600	Mesophilic	100	Screw press	1,800
Type - reactors volume (m3)	%Total solids in digester	Type of valorization	Biomethane injected (Nm <sup>3</sup> /hour)	V <sub>CH<sub>4</sub></sub> produced (Nm <sup>3</sup> /year)	CH <sub>4</sub> yield (Nm <sup>3</sup> CH <sub>4</sub> /ton feedstock)
Digester - 2000 Post-dig. - 2000 Storage tank - 6000	12	Upgrading	145	1,189,000	87.4

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Table 1: Agricultural plant features

156 The two Basidiomycetes fungus strains were ordered from Mycelia bvba (Deinze, Belgium), a  
 157 professional spawn and mycelium culture laboratory. Strain M2191 of *Pleurotus Ostreatus*, also called  
 158 winter oyster mushroom, and strain M5012 of *Stropharia rugoso-annulata*, also called garden giant  
 159 mushroom, were received under spawn form. For *PO*, spawn consisted in millet seeds colonized by  
 160 mycelium while for *SRA*, spawn colonized wheat straw. Both were stored at 4 °C until experiments  
 161 started.

## 162 2.2. Cultivation method

163 An isolated wooden box was designed. Inside the box, a system using a vacuum pump KNF Laboport™  
 164 (Freiburg-im-Breisgau, Germany) bubbling air in vessels containing water ensured efficient air  
 165 humidification. Temperature within the box was lowered and regulated using a Minichiller Huber  
 166 (Offenburg, Germany) and piping system. Temperature and air relative humidity inside the box were  
 167 measured via a Hygrasgard® RPFTF – 20 – Modbus sensor from S+S Regeltechnik (Nürnberg, Germany)  
 168 and values were recorded every 10 minutes via a software developed internally. During experiments,  
 169 relative humidity was maintained close to 75% while box temperature varied between 20-25°C as a  
 170 function of the variation of the external temperature with day-night cycle. Finally, the box could be  
 171 closed ensuring a dark environment. These conditions were inside the range for good substrate  
 172 colonization according to both Mycelia bvba strains factsheets and previous studies [24,25].

## 173 2.3. Screening of solid-state fermentation conditions

174 Screening was performed to determine SSF conditions on SD of *Pleurotus ostreatus*. Two conditions  
 175 were applied to SD before inoculation: (i) no treatment; (ii) quicklime (addition of calcium oxide, CaO)  
 176 at 2% w/w and then autoclave for 30 minutes at 121 °C. Following these treatments, four spawn levels  
 177 were tested: 5, 10, 15 and 20% w/w of SD. Fungal SSF was carried out by mixing 10 grams of SD,  
 178 potentially treated, with the corresponding amount of *PO* spawn within Corning-Gosselin® 40mL  
 179 polypropylene tubes (Borre, France). A control, where SD was replaced by moisturized and sterilized  
 180 wheat straw, was added to be sure that *PO* spawn was active. Tubes were then placed inside the box  
 181 in the dark. After 10 days, colonization inside the tubes, due to SSF, were visually assessed according

182 to the following scale: (0) no colonization at all; (+) some area of SD are colonized; (++) SD is totally  
 183 colonized. Selected SSF conditions for *PO* were similarly applied to *SRA*.

#### 184 2.4. Evaluation of optimal fungal solid state fermentation duration

185 Selected conditions for efficient SSF were applied, which consisted of 2% w/w CaO and 20 minutes  
 186 autoclaving at 121 °C of SD followed by a 20% w/w spawn addition. Following inoculation, different  
 187 SSF times under aerobic conditions were evaluated. For *PO*, five durations were tested: 5.5, 7.5, 10, 15  
 188 and 21 days. The first four ones reach the incubation/colonization phase, while the latter is in the  
 189 fructifying phase. In that case, the wooden box was slightly opened after day 15 to ensure half-light  
 190 and trigger fruiting. For *SRA*, three durations were tested: 5.5, 7.5 and 15 days. Two controls were also  
 191 added, consisting in: (i) untreated SD, *PO* spawn and *SRA* spawn left under aerobic conditions inside  
 192 the wood box for 5.5 and 15 days (ii) SD quicklimed at 2% w/w limed and autoclaved for 20 minutes,  
 193 left under aerobic conditions inside the wooden box for 5.5 and 15 days. A summary of the  
 194 experimental set-up is given in **Table 2**. For every 16 conditions, Corning-Gosselin® 40 mL tubes  
 195 containing 2 grams of VS of corresponding substrates were prepared and placed for the given duration  
 196 inside the wooden box that was subsequently closed.

Aeration duration	5.5 days	7.5 days	10 days	15 days	21 days
Tested substrates					
Untreated SD	x3			x3	
CaO + autoclaved SD	x3			x3	
<i>PO</i> spawn untreated	x3			x3	
<i>SRA</i> spawn untreated	x3			x3	
<i>PO</i> spawn + treated SD	x3	x3	x3	x3	x3
<i>SRA</i> spawn + treated SD	x3	x3		x3	

197 **Table 2: Experiments performed to evaluate optimal fungal SSF duration**

#### 198 2.5. Physico-chemical and microbial analysis performed on solid digestate and mycelia

199 To characterize the different substrates several methods were used. Total Solids (TS) and VS contents  
 200 were obtained following the standard methods of the American Public Health Association [26]. OM  
 201 corresponds to VS, therefore for the rest of the article VS term will be used to describe OM. An  
 202 AutoKjehdahl Unit K-370, BUCHI (Flawil, Switzerland) was used to determine the total Kjeldahl  
 203 nitrogen. Total carbon content was measured using a Shimadzu TOC-VCSN Analyzer (Kyoto, Japan)  
 204 coupled to a Shimadzu solid sample module SSM-5000A. The pH was measured using a Mettler  
 205 Toledo Seven S2-meter with an InLab® Expert Pro-ISM sensor (Columbus, US). The chemical oxygen  
 206 demand (COD) of solid samples was performed using an Aqualytic 420721 COD Vario Tube Test MR 0–  
 207 1500 mg·L<sup>-1</sup> (Dortmund, Germany). Samples were first freeze-dried and then ground using a Retsch  
 208 mixer mill MM 200 (Haan, Germany) and associated stainless steel grinding jar. Vibrational frequency  
 209 was fixed at 25 Hz·min<sup>-1</sup>·g<sup>-1</sup> of material in the jar in order to obtain a homogenous powder between  
 210 samples. 0.25 grams of sample powder were poured into 10 mL of 98% w/w H<sub>2</sub>SO<sub>4</sub> and set under  
 211 strong agitation overnight to solubilize solid particles. Dilution with MilliQ water up to 250 mL allowed  
 212 pipetting. 2 mL of sample adequately diluted were then pipetted into each tube. Oxidation reactions  
 213 in tubes were performed in a HACH COD reactor at 150 °C for 2 hours (Loveland, US). COD  
 214 concentrations were measured using an Aqualytic MultiDirect spectrophotometer. To determine

215 microbial richness of untreated solid digestate, a bacterial quantitative Polymerase Chain Reaction  
216 (qPCR) was carried out following Braun et al., (2011) procedure.

## 217 **2.6. Respiration assessment**

218 VS loss due to respiration of fungi or endogenous SD microorganisms inside the wooden box was  
219 evaluated using TS/VS measurement. Initial VS in each tube was known. At the end of each post-  
220 treatment, 3 tubes were first placed at 105 °C to determine TS. Dry samples were then entirely  
221 transferred into ceramic crucibles and placed at 550 °C to determine remaining VS contents. Difference  
222 between the initial VS and the remaining VS content corresponds to VS loss from respiration.

## 223 **2.7. Determination of methane yield and biodegradability**

224 Biomethane Potential (BMP) tests were performed to simulate recirculation of SD within the biogas  
225 plant after post-treatment. For each condition of **Table 2**, BMP tests were carried out in triplicate and  
226 prepared similarly to Monlau et al., (2012). Briefly, for each tube containing tested substrates, the  
227 matter was mixed with a macroelement solution, an oligoelement solution, a bicarbonate buffer as  
228 well as an anaerobic sludge at 5 g<sub>VS</sub>·L<sup>-1</sup>, before being incubated under agitation at 35 °C. Biogas  
229 production was monitored twice a week at the beginning and less frequently as production slowed  
230 down. Biogas volume was measured using a Keller LEO 2 digital manometer (Winthethur, Switzerland)  
231 and biogas composition was determined using a PerkinElmer® Clarus 580 gas chromatograph  
232 (Waltham, US) equipped with two Restek columns (Bellefonte, US): the first (RT®-Q-Bond) was used to  
233 separate CO<sub>2</sub> from other gases, the second (RT®-Msieve 5A) was used to separate O<sub>2</sub>, N<sub>2</sub>, CH<sub>4</sub>, and H<sub>2</sub>.  
234 The carrier gas used was argon delivered at 350 kPa and 35 mL·min<sup>-1</sup> into the column. Oven, injector  
235 and detector temperatures were set respectively at 60 °C, 250 °C and 150°C. A thermal conductivity  
236 detector was used for gas detection. According to standardized practices, for each condition,  
237 endogenous methane production was considered by subtracting the gas generated in the controls  
238 (inoculum only) and BMP tests were stopped when daily methane production during three consecutive  
239 days was <1% of the accumulated volume of methane [29].

240 Methane yield (in mL CH<sub>4</sub>·g<sup>-1</sup> VS initial) is defined as the accumulated amount of methane produced as  
241 a function of the initial VS content of the sample before post-treatment (≈2 g of VS), while  
242 biodegradability (in mL CH<sub>4</sub>·g<sup>-1</sup> VS final) is defined as the methane produced by the remaining matter.  
243 Biodegradability was expressed as a function of the final VS content of the sample after the post-  
244 treatment and respiration (less than 2 g of VS). BMP values obtained were compared to two types of  
245 control: (i) direct codigestion: untreated SD and spawn are directly anaerobically digested without  
246 contact time and (ii) treated codigestion: controls where treated SD (lime and autoclaving) and spawn  
247 are separately stored under aerobic conditions for 5.5 or 15 days before being anaerobically digested  
248 without contact time. In both cases, proportions between SD and spawn corresponded to identical VS  
249 ratios obtained in SSF trials with 20% w/w spawn levels. Overall BMPs for the controls were obtained  
250 as follows:

$$251 \quad BMP_{direct\_codigestion} = BMP_{Spawn} * \%g VS_{Spawn} + BMP_{untreated\_dig} * \%g VS_{dig} \quad (1)$$

$$252 \quad BMP_{treated\_codigestion\_Xdays} = BMP_{aerated\_spawn\_Xdays} * \%g VS_{spawn} + BMP_{treated\_dig\_Xday} * \%g VS_{dig} \quad (2)$$

253



## 254 2.8. Volatile solids characterization

255 VS was characterized via sequential chemical extractions according to Jimenez et al., (2015) protocol.  
256 Firstly, selected samples were freeze-dried and ground to obtain fine and homogenous powders.  
257 Subsequent sequential extractions were performed on 0.5 g of sample. Four VS fractions of decreasing  
258 accessibility were then obtained by applying increasingly strong chemical solutions: (i) soluble  
259 extractable OM (SPOM) using  $\text{CaCl}_2$  (10 mM), (ii) readily extractable OM (REOM) using NaOH (10 mM)  
260 + NaCl (10mM), (iii) slowly extractable OM (SEOM) using NaOH (0.1M) and (iv) poorly extractable OM  
261 (PEOM) using  $\text{H}_2\text{SO}_4$  72% w/w. At each step, the solubilized VS was recovered in the supernatant by  
262 centrifugation (18,750 g for 20 min at 4 °C) and filtered at 0.45  $\mu\text{m}$ . VS of each fraction was then  
263 characterized via COD measurement. Finally, the non-extractable OM (NEOM) was calculated by  
264 subtracting the four fractions of VS extracted from the sample from the total VS. This total VS being  
265 obtained by measuring COD on the initial powder sample using the previously described COD method  
266 for solids (see 2.5.). Here, it can be specified that SPOM corresponds to soluble proteins and sugars,  
267 REOM and SEOM to recalcitrant proteins, lipids and some humic acids, PEOM to holocelluloses and  
268 finally NEOM to lignin-like molecules and complex humic acids. This characterization method was  
269 preferred to other existing methods of lignin quantification [31,32], as SD, contrary to crops or woody  
270 biomass, not only contains lignin but also complex humic acids that are not measured using these  
271 methods.

272 On some samples, the fluorescence spectra of liquid extracts were recorded on a Perkin Elmer LS55  
273 and a complexity ratio was calculated according to Jimenez et al., (2015). This index is defined as the  
274 ratio of the sum of the fluorescence volumes of the most complex molecules (lignin, humic acid...) over  
275 the sum of the fluorescence volumes of the protein-like molecules.

276 Finally, correlations between the different VS fractions, methane yields and biodegradability were  
277 analyzed via a principal component analysis (PCA). PCA was performed using SIMCA software from  
278 UMETRICS (Umeå, Sweden).

## 279 2.9. Evaluation of the impact of post-treatments and solid digestate recirculation on plant 280 methane yield compensation

281 To determine the extent to which SD recirculation can compensate a feedstock shortage or be  
282 integrated in the ration ( $\gamma_{\text{CH}_4\text{ yield compensation}}$ ), we used the following equation:

$$283 \gamma_{\text{CH}_4\text{ yield compensation}} = \frac{\text{Corrected\_BMP}_{SD} * \%VS_{SD} * M_{SD\text{ recirculated}}}{V_{\text{CH}_4\text{ produced yearly}}} \quad (3)$$

284  $\text{Corrected\_BMP}_{SD}$  (in  $\text{Nm}^3 \text{CH}_4 \cdot \text{ton}^{-1} \text{VS}$ ) aimed to simulate full-scale methane production value. It was  
285 obtained by applying a 0.8 correction factor to obtain BMP values according to Holliger et al. study  
286 [33].  $\%VS_{SD}$  corresponds to the ratio of volatile solids measured at lab scale over fresh matter of SD.  
287  $M_{SD\text{ recirculated}}$  (tons/year) corresponded to 20%, 50% and 80% of the total SD produced yearly (1800  
288 tons/year). We assume that it is not possible to recirculate 100% of the SD as some minerals and  
289 recalcitrant organic matter need to exit the system notably to avoid AD inhibitions and be used for  
290 land fertilization. For fungal post-treatment calculations, 20%w inoculum was added to  $M_{SD\text{ recirculated}}$   
291 and  $\%VS_{SD}$  of the mix was calculated based on initial VS content of SD as well as inoculum (21% for

292 PO+SD and 17% for SRA+SD). Besides, methane yields taking into account VS losses were used. At last,  
 293 the volume of methane produced yearly as previously calculated corresponded to 1,189,000 Nm<sup>3</sup>.

294 To determine a number of tons of feedstocks ( $\Delta_{feedstock}$ ) that would be replaced by such strategy, we  
 295 applied equation (4), where CH<sub>4</sub> yield corresponds to the average methane production per ton of  
 296 feedstock (84.9 Nm<sup>3</sup> CH<sub>4</sub>·ton<sup>-1</sup> feedstock):

$$297 \quad \Delta_{feedstock} = \frac{Corrected\_BMP_{SD} * \%VS_{SD} * M_{SD\ recirculated}}{CH_4\ yield} \quad (4)$$

298 Finally, to estimate the impact of recirculating SD on digester TS (Digester\_TS<sub>recirculation\_SD</sub>), we used the  
 299 equation herein below:

$$300 \quad Digester\_TS_{recirculation\_SD} = \frac{(M_{feedstock} - \Delta_{feedstock}) * \%TS_{digester} + M_{SD\ recirculated} * \%TS_{SD}}{M_{feedstock} - \Delta_{feedstock} + M_{SD\ recirculated}} \quad (5)$$

301 In this equation, the amount of feedstock ( $M_{feedstock}$ ) corresponds to the initial ration of the digester  
 302 (14,000 tons/year) and initial TS content of the digester was 12% ( $\%TS_{digester}$ ). Digester\_TS<sub>recirculation\_SD</sub>  
 303 can be defined as the new TS content of the digester, following implementation of the SD recirculation  
 304 strategy.

### 305 **3. Results and discussion**

#### 306 **3.1. Substrates features**

307 Several substrate factors are known to affect mushroom growth such as C/N ratio, moisture, pH,  
 308 microbial load and spawn level [34]. SD was characterized in order to determine if it was a suitable  
 309 substrate for fungus growth (**Table 3**). Moisture and pH were slightly higher than what is usually stated  
 310 as optimal for growth. In the case of *Pleurotus* species: (i) moisture contents between 50-75% are often  
 311 targeted as higher levels can favor fungus diseases and microbial competition and (ii) an initial pH of  
 312 6.5-8.7 is preferred [34,35]. However, high nitrogen content (C/N ratio below 40 in the case of  
 313 synthetic medium) was identified as a potential way to enhance ligninolytic activities of *Pleurotus*  
 314 species, which was relevant for the tested post-treatment [36]. It can be noticed that the pH of spawn,  
 315 also characterized in **Table 3**, were around 5.5 due to the fact that mycelium colonization led to an  
 316 acidification of the bulk growth substrate. Initial trials at different spawn levels (5-20% w/w) for *PO* did  
 317 not lead to any significant colonization of untreated SD. This can be seen in **Fig. 1**. This is not due to a  
 318 problem in *PO* spawn activity, as wheat straw control was partly colonized. Treatment of the SD is thus  
 319 necessary to allow its colonization by *PO*.

Substrate	%TS	%VS	%C·g <sup>-1</sup> TS	%N·g <sup>-1</sup> TS	C/N	pH
Solid Digestate (SD)	20.5 ±0.3	15.5 ±0.1	33.2 ±0.2	1.7 ±0.1	19.3	9.1
SD quicklimed & autoclaved	22.9 ±0.2	15.2 ±0.1	32.4 ±0.2	1.4 ±0.1	23.6	11.4
<i>PO</i> spawn	48.9 ±0.1	43.1 ±1.5	39.7 ±0.3	2 ±0.1	19.6	5.7
SRA spawn	26.7 ±0.5	24.3 ±0.4	31.2 ±0.3	1.4 ±0.1	22.9	5.4

320 **Table 3: Substrates features**

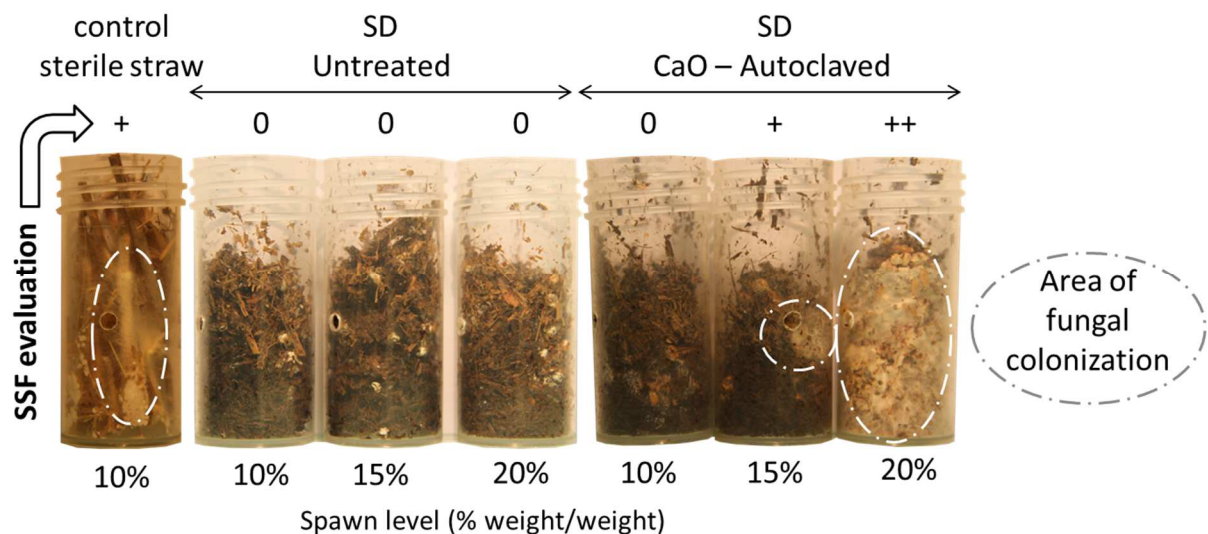
#### 321 **3.2. Determination of fungal SSF conditions**

322 Sterilization appears to be the most important parameter to work on as the microbial load of SD was  
 323 high and *PO* is reported to be sensitive to bacteria competition during its development [37]. Results of

324 the qPCR on untreated SD displayed an absolute abundance of  $7.3 \cdot 10^{11}$  ( $\pm 3.9 \cdot 10^{11}$ ) numbers of 16S  
 325 gene copies per gram of fresh matter (FM). This corresponds to an average of  $1.8 \cdot 10^{11}$  bacterial cells·g<sup>-1</sup>  
 326 FM, which is in the range of values found in literature for similar digestates coming from AD plants  
 327 (Braun et al., 2011). However, this amount is high in comparison to other growth media for fungi such  
 328 as bulk soil ( $10^8$  cells·g<sup>-1</sup>) or even unsterilized straw ( $10^9$  cells·g<sup>-1</sup>) [38,39].

329 The sterilization strategy was based on the combination of an alkaline and thermal treatment to reduce  
 330 microbial load and activity and results are displayed in **Fig. 1**. Indeed, alkaline conditions appear to  
 331 reduce activity of competing microorganisms, while mycelium development of *PO* is not greatly  
 332 affected [35,40]. Therefore, a 2% w/w quicklime powder was used to alkalinize SD as it is a cheap  
 333 chemical, generally used on farms for agricultural practice, it is more concentrated than slacked lime  
 334 previously successfully used by Hernandez et al., (2003) and it has an additional delignification effect  
 335 (Ramos-Suárez et al., 2017). Autoclaving was subsequently performed to reduce microbial load before  
 336 inoculation. Following this treatment, moisture was close to 75%, C/N ratio increased slightly, as  
 337 nitrogen probably volatilized under ammonia form during autoclaving, and pH was alkaline (see  
 338 **Table 3**.). Only a high 20% w/w of *PO* spawn succeeded in colonizing efficiently SD after 10 days of SSF,  
 339 a white coating was formed around the SD. According to these results, selected growth conditions for  
 340 *PO* and *SRA* consisted in addition of quicklime (2% w/w), autoclaving and a 20% w/w spawn level. Full-  
 341 scale feasibility of such a strategy will be discussed thereafter.

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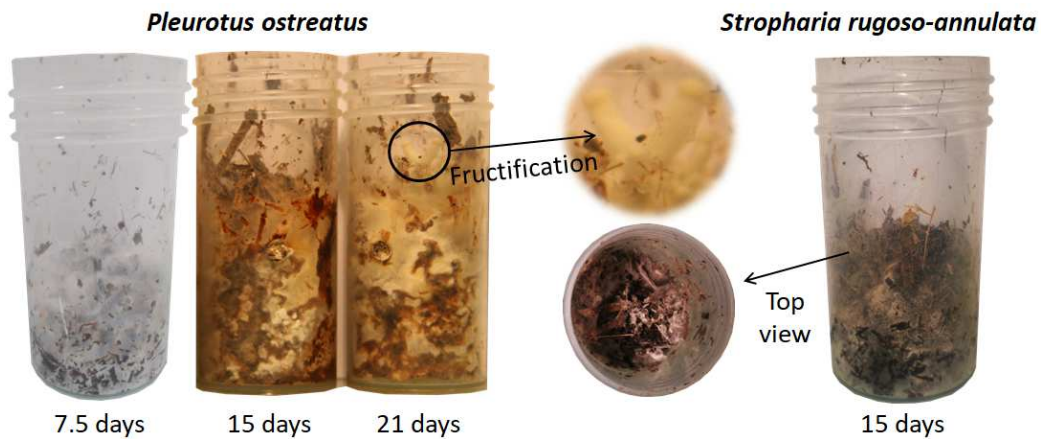
345

**Figure 1: Screening of colonization conditions during SSF of *Pleurotus ostreatus* on solid digestate and visual assessment according to predefined evaluation scale (5% spawn level is not shown as no colonization occurred).**

346

### 3.3. Impact of fungal SSF duration on volatile solids loss

347 Selected growth conditions led to colonization of SD by *PO* during SSF. For each SSF duration, from 5.5  
 348 to 21 days, all tubes had successful colonization with a mycelium that tightens and forms an  
 349 increasingly strong coating around the SD in time. Some tubes at 21 days even showed some  
 350 fructification starting with small carpophores growth at the top of SD. For SSF of *SRA*, growing and SD  
 351 colonization was less obvious than for *PO*. At 5.5 and 7.5 days colonization was not significant at sight,  
 352 and at 15 days some white mycelium had developed but it was not comparable with *PO* colonization.  
 353 To illustrate these observations, some tubes are shown in **Fig. 2**.



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Figure 2: Photographs of solid digestate colonized by *PO* and *SRA* after SSF at different times

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The trend of VS loss for each condition was plotted in time in **Fig. 3**. VS is lost in time for every condition except at 5.5 days in the case of quicklimed and autoclaved SD (thermo-alkaline post-treatment). This is probably due to an efficient sterilization and alkaline conditions that suppressed microbial aerobic activity for a few days. However, at 15 days, thermo-alkaline post-treatment led to a VS loss higher than the SD only aerated (19% of VS loss against 12.8%). This is probably due to the fact that this treatment enhanced the amount of easily accessible VS that can be subsequently respired. Aerated spawn controls show VS losses between 13.3-23.5% after 15 days, which can be explained by further colonization and respiration by *PO* and *SRA*, respectively. For *SRA* colonizing SD, VS loss at 5.5 days was higher than the control, probably due to some colonization of SD by *SRA*. Nevertheless, after 15 days, VS loss was similar to the control showing that there were no specifically strong interactions between SD and *SRA* over time. For *PO*, VS loss was always higher than the control reflecting the colonization of the SD by *PO* and a higher activity. VS loss reaches around 20% after 15 days. Similar VS loss was observed in studies using fungi to pretreat biomass before AD [42–44]. VS loss directly impacts methane yield since the VS consumed for respiration is no longer available to be converted to methane.

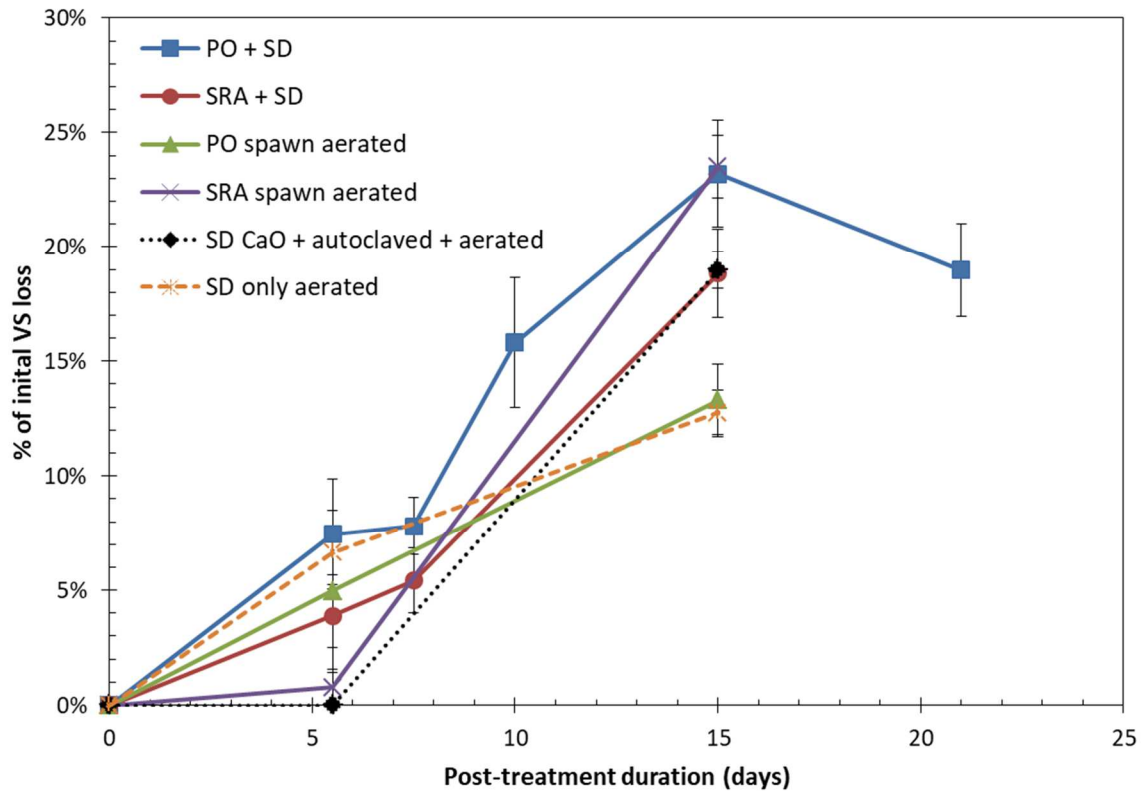


Figure 3: Volatile solids loss in time for the different conditions applied to SD

### 3.4. Impact of fungal SSF duration on methane yield and biodegradability

Results of *PO/SRA* SSF, thermo-alkaline post-treatment and simple aeration of spawn or SD on methane yield and biodegradability are gathered in **Table 4**. Several observations can be drawn from that: (i) Methane yield of SD, *SRA* spawn and *PO* spawn only aerated were respectively around 10%, 20% and 30% lower than their corresponding control (no aeration). VS loss was not compensated by a gain in biodegradability, which significantly decreased for *PO* (by 25%) at 5.5 and 15 days. This is also the case at 5.5 days for *SRA* (by 17%) and SD (by 7.6%), but biodegradability was not significantly different after 15 days. One hypothesis is that endogenous SD microorganisms or *PO* and *SRA* used VS which was easy to access for their growth/metabolism during this period which can explain the loss in biodegradability generally observed. Lignin-like molecules were not specifically degraded.

(ii) Thermo-alkaline post-treatment followed by a short 5.5-day aeration displayed an enhanced methane yield and biodegradability of 13% in comparison to untreated SD. It is likely that this kind of post-treatment acted on VS biodegradability and notably lignin as it was recently observed by Mustafa et al., (2018) on sugarcane bagasse. After 15 days, similarly to only aerated SD, methane yield was significantly lower (15%) and biodegradability was not significantly enhanced. It is likely that endogenous or/and exogenous microorganisms, after a lag time due to sterilization and increased pH, used the additional easy to degrade VS fraction that was released during post-treatment for their growth, explaining the biodegradability decline.

(iii) For *PO*, methane yield was always lower in comparison to direct codigestion (untreated SD and *PO* spawn). The longer the SSF time, the lower the methane yield, with up to 50% methane yield loss after 21 days SSF. Besides, codigestion of separately treated SD (CaO, autoclaving and 5.5 or 15 aeration

394 days) and *PO* spawn (5.5 or 15 aeration days) both at 5.5 and 15 days displayed higher methane yields.  
395 This means that the interaction between *SD* and *PO* during colonization increased methane loss;  
396 probably due to respiration of *VS* coming from *SD* by *PO*. Colonization by *PO* of *SD* does not appear  
397 beneficial as biodegradability is decreased over time, probably reflecting no specific ligninolytic activity  
398 of *PO* on *SD*. Similar results were observed by Rouches et al., (2015) for certain fungi strains. In their  
399 study, methane yield of pretreated wheat straw was decreased due to carbohydrate consumption by  
400 fungi.

401 (iv) For *SRA*, short term aeration (5.5 and 7.5 days) did not lead to any significant methane yield loss.  
402 However, at 15 days methane yield was decreased by 16.8% as *VS* loss occurred without any significant  
403 biodegradability increase. Again, *SRA* interaction with *SD* is not clearly shown as methane yield loss is  
404 similar to the codigestion of *SRA* spawn and treated *SD* aerated 15 days separately. The lack of  
405 colonization can be due to suboptimal conditions as *SRA* is generally growing on straw, so a pH around  
406 6.5-7.5 and C/N ratio of 50-100 is preferred compared to 11.4 and 24 in our case, respectively. Besides,  
407 growth rate is slower than *PO* and 15 days might not be long enough to see a clear colonization [47].

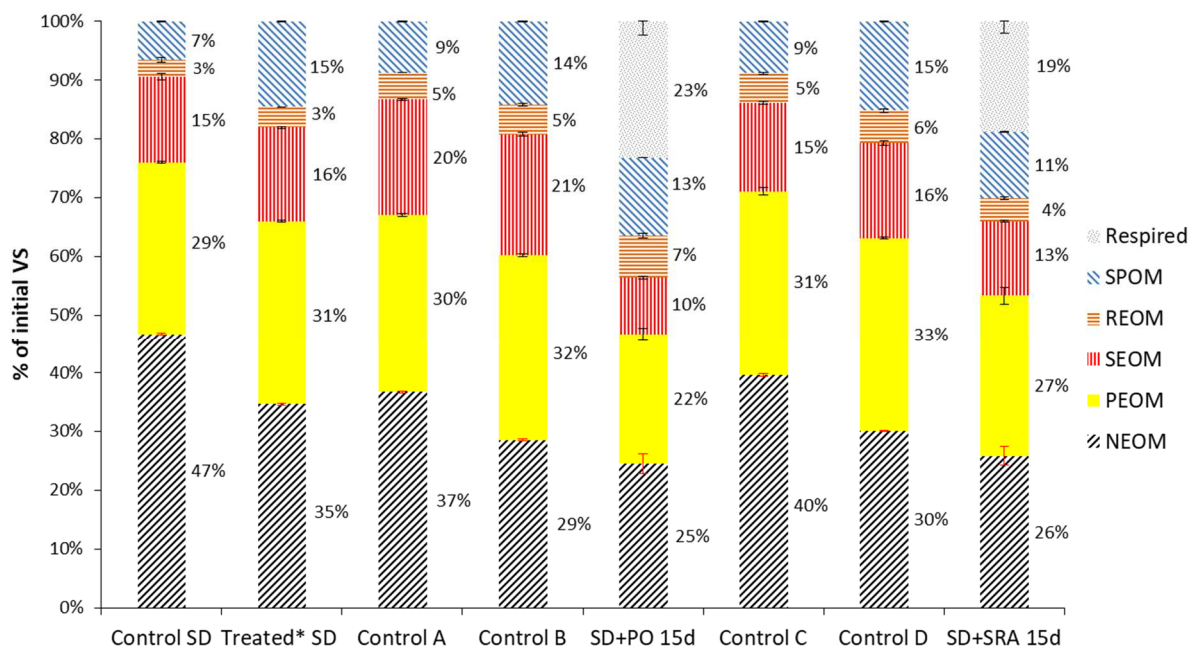
	Sample	Aeration duration	VS loss (% initial VS)	Methane yield (mL CH <sub>4</sub> ·g <sup>-1</sup> initial VS)	% Methane yield variation	Biodegradability (mL CH <sub>4</sub> ·g <sup>-1</sup> final VS)	% Biodegradability variation
Treated SD	<b>SD control</b>	<b>0</b>	<b>0</b>	<b>157 (±3)</b>	<b>/</b>	<b>157 (±3)</b>	<b>/</b>
	SD Cao autoclaved aerated	5.5	0 (±0.3)	177 (±2)	13	177 (±2)	+13
	SD Cao autoclaved aerated	15	19 (±0.8)	133 (±4)	-15.2	158 (±5)	Not significant
	SD only aerated	5.5	6.6 (±1)	136 (±5)	-13.3	145 (±5)	-7.6
	SD only aerated	15	12.8 (±1)	142 (±6)	-9.6	160 (±6)	Not significant
Aerated spawn	<b>PO spawn control</b>	<b>0</b>	<b>0</b>	<b>365 (±30)</b>	<b>/</b>	<b>365 (±30)</b>	<b>/</b>
	PO spawn aerated	5.5	5 (±3.5)	258 (±12)	-29.3	271 (±13)	-25.8
	PO spawn aerated	15	13.3 (±1.6)	238 (±3)	-34.8	269 (±4)	-26.3
	<b>SRA spawn control</b>	<b>0</b>	<b>0</b>	<b>203 (±6)</b>	<b>/</b>	<b>203 (±6)</b>	<b>/</b>
	SRA spawn aerated	5.5	6.5 (±0.8)	158 (±8)	-22.2	168 (±8)	-17.2
	SRA spawn aerated	15	23.5 (±1.4)	160 (±5)	-21.2	197 (±6)	Not significant
Treated SD + PO	<b>Direct codigestion SD + PO</b>	<b>0</b>	<b>0</b>	<b>232 (±12)</b>	<b>/</b>	<b>232 (±12)</b>	<b>/</b>
	Codigestion treated separately	5.5	2 (±1.4)	210 (±6)	-9.5	215 (±6)	Not significant
	SD + PO	5.5	7.5 (±2.4)	174 (±8)	-25	187 (±9)	-19.4
	SD + PO	7.5	7.8 (±1.3)	170 (±5)	-26.7	183 (±5)	-21.1
	SD + PO	10	15.8 (±2.8)	143 (±14)	-38.4	166 (±16)	-28.4
	Codigestion treated separately	15	17.2 (±1)	197 (±4)	-15.1	207 (±4)	-10.8
	SD + PO	15	23.2 (±2.3)	149 (±13)	-35.8	184 (±16)	-20.7
	SD + PO	21	19 (±2)	114 (±5)	-50.9	136 (±6)	-41.4
Treated SD + SRA	<b>Direct codigestion SD + SRA</b>	<b>0</b>	<b>0</b>	<b>167 (±3)</b>	<b>/</b>	<b>167 (±3)</b>	<b>/</b>
	Codigestion treated separately	5.5	1.5 (±0.2)	173 (±3)	Not significant	175 (±3)	+3.6
	SD + SRA	5.5	3.9 (±1.4)	158 (±9)	Not significant	164 (±10)	Not significant
	SD + SRA	7.5	5.4 (±1.4)	169 (±8)	Not significant	178 (±9)	Not significant
	Codigestion treated separately	15	19.9 (±0.9)	139 (±4)	-16.8	166 (±5)	Not significant
	SD + SRA	15	18.8 (±1.9)	139 (±7)	-16.8	165 (±9)	Not significant

Table 1: Volatile solids loss and BMPs results of solid digestate post-treatment

409 **3.5. Volatile solids fractions: evolution following post-treatments**

410 To better understand the impact of these different treatments on VS, fractionation of 8 samples was  
 411 carried out and results were gathered in **Fig. 4**. Impact of fungal treatment on VS was evaluated at 15  
 412 days as methane yield was lower both for *PO* and *SRA*. Controls A and C, which correspond to direct  
 413 codigestion of untreated SD and spawn, allow to have an insight into VS evolution after thermo-  
 414 alkaline treatment, aeration and fungal colonization. Controls B and D correspond to codigestion of  
 415 thermo-alkaline post-treated SD and untreated spawn. They enable to distinguish the effect of the  
 416 thermo-alkaline treatment from aeration and fungal colonization.

417 Firstly, untreated SD is in range with previously analyzed agricultural SD using the same method,  
 418 notably with a NEOM close to 45% showing that agricultural SD can accumulate a large quantity of  
 419 hardly degradable molecules such as lignin [48]. Thermo-alkaline post-treatment (Treated\* SD in **Fig.**  
 420 **4**) had a strong effect on NEOM reducing it by 26% while SPOM content was increased by 123% (see  
 421 **Table 5**). Fluorescence spectroscopy analysis was used to have insight into the SPOM composition and  
 422 the complexity ratio was slightly increased by 8% (from 1.42 to 1.54) due to higher lignin-like and humic  
 423 acid-like compounds. This validates our previous hypothesis and indicates that NEOM get more  
 424 accessible and potentially released soluble compounds as well as embedded holocelluloses explaining  
 425 the 13% increase in methane yield. Mustafa et al., (2018) similarly obtained a delignification of 46%  
 426 and a decrease in hemicellulose of 83% by a combination of a hydrothermal treatment at 180 °C and  
 427 addition of 8.5% Ca(OH)<sub>2</sub> on sugarcane bagasse. On rice straw, 5% Ca(OH)<sub>2</sub> combined with 6 hours 80°C  
 428 thermal treatment also lead to 31% lignin and 15% hemicellulose content reduction, as well as a 25%  
 429 biogas production increase [49].



430 **Figure 4: Distribution of the different VS fractions in percentage of initial VS for various samples. (Treated\* SD) SD CaO +**  
 431 **autoclave + 5.5 aeration days; (Control A) untreated SD and PO; (Control B) Treated\* SD + untreated PO; (Control C)**  
 432 **untreated SD and SRA; (Control D) Treated\* SD + untreated SRA**  
 433  
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435



	SPOM	REOM	SEOM	PEOM	NEOM
Thermo-alkaline post-treatment	+123%	+22%	+9%	+7%	-25,77%
<i>PO</i> colonization	-7%	+44%	-53%	-30%	-14%
<i>SRA</i> colonization	-26%	-28%	-21%	-17%	-14%

436 **Table 5: Impact of treatments on the evolution of the different fractions of the VS in comparison to the untreated SD**

437 Control A and C have lower NEOM contents than SD which can be explained by the fact that *PO* and  
438 *SRA* spawns had lower contents of lignin-like molecules. Between Control A and B as well as C and D,  
439 decrease in NEOM contents and increase in SPOM contents are due to the thermo-alkaline post-  
440 treatment on SD which degrades lignin-like molecules into soluble ones. When comparing control B  
441 (treated SD and untreated *PO*) to the colonized digestate with *PO*, it can be observed in **Table 5** that  
442 *PO* activity was not really selective toward lignin (only -14% for NEOM) but rather proteolytic (-53% for  
443 SEOM) and hemicellulolytic (-30% for PEOM). This non-specific activity can explain the measured loss  
444 in biodegradability and in methane yield. Similarly, for *SRA*, activities were not specific as all VS  
445 fractions were used (between -14% and -28%). Contrary to *PO*, SPOM and REOM fractions were  
446 degraded up to 25%. This could be linked to the activity of endogenous microorganisms such as  
447 proteolytic bacteria. It might indicate that *SRA* probably did not entirely colonize SD and is still  
448 competing or coexisting with endogenous SD flora.

449 A hypothesis to explain low lignin selectivity of fungi strains could come from the sterilization process  
450 (thermo-alkaline post-treatment) that released soluble compounds such as sugar, which can be then  
451 preferentially used by fungi. It has been previously shown that the addition of glucose can limit  
452 delignification by fungi [46]. Besides, to our knowledge, it is the first time that effects of post-  
453 treatments, different from composting, on SD lignin-like content are clearly evaluated using the  
454 fractionation method. This method seems particularly adapted to better understand the effect of  
455 various post-treatments on SD.

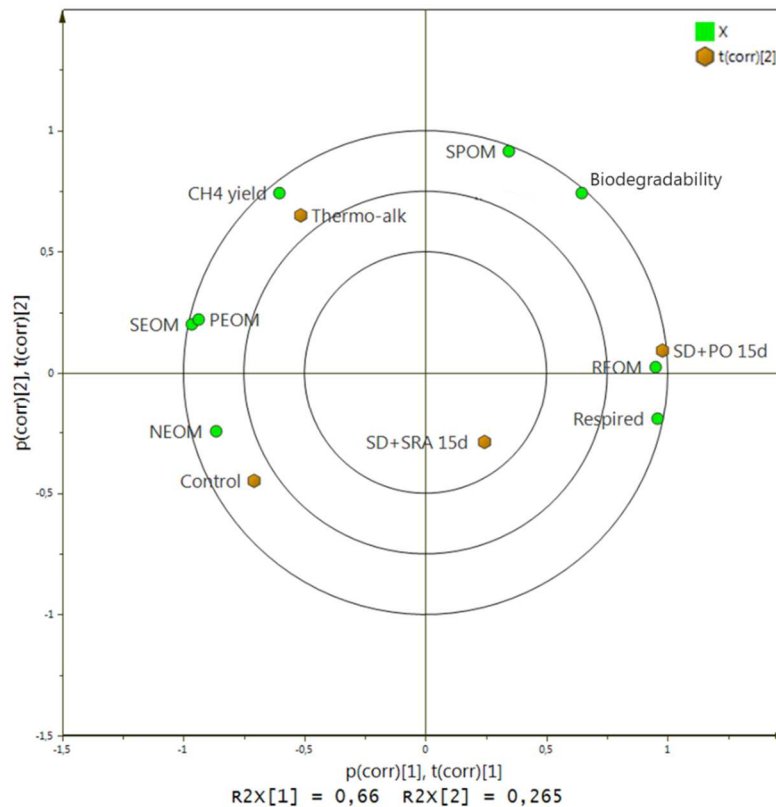
### 456 **3.6. Correlations between VS fractions and methane yield/biodegradability**

457 Data on methane yield and biodegradability from **Table 4** as well as VS fraction percentages from **Fig.**  
458 **4**, for control SD, CaO+autoclave+5.5d aeration SD, SD+PO 15d aeration and SD+SRA 15d aeration  
459 samples, were used to perform a PCA analysis presented in **Fig. 5**. Looking at the PCA representation,  
460 several observations can be made.

461 Only two major components are sufficient to describe 92.5% of the data variability. Component 1  
462 explains 66% of the total variability. It can be shown that SD biodegradability is negatively correlated  
463 with the NEOM content. Anaerobic biodegradability of lignin-like molecules are reported to be low,  
464 therefore this correlation appears to make sense. SD control which has the highest lignin content (47%  
465 initial VS) has logically the lowest biodegradability (157 mL CH<sub>4</sub>·g<sup>-1</sup> final VS). Besides, SPOM content  
466 appears to be positively correlated with SD biodegradability. On that point, similar observations were  
467 already found in recent studies, pointing out that a significant positive correlation exists between  
468 soluble content and BMP values [48,50]. For methane yield (once VS loss is taken into account), it can  
469 be shown that it is negatively correlated with respiration. Indeed, during respiration, VS are lost that  
470 would otherwise lead to additional methane production.

471 Regarding these results, a conclusion that can be drawn is that SD post-treatment that aims to improve  
472 methane yield SD should avoid too important VS losses due to aeration and increase soluble molecules

473 (SPOM) content while reducing lignin-like (NEOM) content. Fungal SSF post-treatment does not follow  
 474 these recommendations.



475  
 476 **Figure 5: Correlation circle (scores) obtained from PCA analysis of control SD, CaO+autoclave+5.5d aeration SD and fungal**  
 477 **post-treated SD (SD+PO 15d & SD+SRA 15d) samples.**

### 3.7. Discussion over SD recirculation and the feasibility of fungal SSF post-treatment

479 **Table 6** gathers calculation results from equation (3) (see section 2.9) for a certain percentage of SD  
 480 recirculated (20% to 80%) and applied for the control SD, the thermo-alkaline post-treated SD, the PO  
 481 15 days post-treated SD and the SRA 15 days post-treated SD. Additionally, equations (4) and (5) were  
 482 applied to control SD recirculation.

483 Firstly, direct recirculation of SD into the digester could compensate up to 2.4% of the total plant  
 484 methane yield in the case of a feedstock shortage or when feedstocks need to be economized. By  
 485 assuming that methane production and TS from feedstock is evenly distributed, SD recirculation could  
 486 replace up to 320 tons of feedstock, while only increasing digester TS content up to 12.8% (+6.75%). In  
 487 a context where prices of feedstocks is increasing due to competition among existing biogas plants,  
 488 such strategy can be a good supporting solution. It has following advantages: reduce costs, increase  
 489 flexibility in feedstock management and enhance biogas plant efficiency.

490 Sterilization post-treatment (CaO 2%w + autoclaving) allows to get a slightly higher methane yield  
 491 compensation, up to 2.6%. However, economic interest remains uncertain due to chemical costs (CaO  
 492 costs around 150 \$·ton<sup>-1</sup>), additional heating energy required as well as the accompanying heating  
 493 structure or device (tank...). Nevertheless, further optimization of this post-treatment may enhance its  
 494 interest (notably lower heating temperature or even no heating).

495 Finally, fungal SSF post-treatment appears to give higher methane production recovery, up to 3.6%,  
 496 but this is due to the addition of 20% inoculum that is also producing methane. Besides, it has been  
 497 shown that higher methane yield could be obtained by direct co-digestion of inoculum and SD without  
 498 SSF colonization. Thus, additional inoculum costs, sterilization process as well as VS losses due to fungal  
 499 respiration make fungal SSF post-treatment non-viable for full-scale agricultural biogas plant.

500 Alternatives to thermo-alkaline sterilization treatment might be explored to reduce costs and  
 501 potentially get a higher specific activity toward lignin-like fraction: (i) Trials performed directly on-site,  
 502 using fresh SD from thermophilic plants would be of interest to determine if long term anaerobic and  
 503 thermophilic conditions can create an available ecological niche in SD for subsequent aerobic  
 504 mesophilic fungal SSF. (ii) Spawn could be propagated on-site by colonizing low-cost substrate such as  
 505 weed plants without sterilization [51] or miscanthus pellets [42] in order to apply higher spawn levels  
 506 at an affordable cost. However, it is likely that extensive labor would counterbalance the economic  
 507 gains. (iii) Explore mild alkaline or acidic treatment without sterilization on SD as lower or higher pH  
 508 may inhibit endogenous bacterial activity while favoring fungal growth [52]. Nevertheless, it is  
 509 foreseeable that optimization of the fungal SSF at full scale will be difficult to manage and remain too  
 510 costly. Tracks that explore production of higher value products than methane (e.g. enzymes,  
 511 biomolecules...) is likely to be the only way to enhance economic profitability and render viable this  
 512 strategy.

	20% SD recirculation	50% SD recirculation	80% SD recirculation
Tons of SD recirculated per year	360	900	1,440
CH <sub>4</sub> <sub>production</sub> offset by direct SD recirculation (% of plant methane yield)	0.6%	1.5%	2.4%
$\Delta$ <sub>feedstock</sub> (tons) for direct SD recirculation	80	200	321
New Digester_TS <sub>recirculation_SD</sub> for direct SD recirculation	12.2%	12.5%	12.8%
CH <sub>4</sub> <sub>production</sub> offset by thermo-alkaline post- treated SD recirculation	0.7%	1.6%	2.6%
Tons of SD + fungal inoculum recirculated per year (80% SD + 20% inoculum)	432	1,080	1,728
CH <sub>4</sub> <sub>production</sub> offset by PO post-treated SD (15d) recirculation	0.9%	2.3%	3.6%
CH <sub>4</sub> <sub>production</sub> offset by SRA post-treated SD (15d) recirculation	0.7%	1.7%	2.7%

513 **Table 6: Evaluation of the effect of various amounts of SD (post-treated or not) recirculated on plant methane yield**  
 514 **compensation and digester TS content.**

#### 515 4. Conclusion

516 In the case of a feedstock shortage or when feedstocks need to be economized it has been shown, for  
 517 the agricultural biogas plant we studied, that direct recirculation of SD into the digester could  
 518 compensate up to 2.4% of the total plant methane yield (equivalent of 320 tons feedstock). It is a low-  
 519 cost strategy that increase flexibility in feedstock management and allow a higher plant methane yield

520 recovery. Concerning fungal solid-state fermentation of SD before recirculation into agricultural biogas  
521 plants, it has never been evaluated until now. It appears not to be a viable strategy to enhance energy  
522 recovery from SD within the tested conditions. For the two strains studied, sterilization and a high  
523 spawn level were needed to ensure fungal colonization. Besides, fungal activities during SSF were not  
524 specific to the most complex fraction, leading to uncontrolled VS losses and subsequent decrease of  
525 biodegradability and methane yield from SD. Overall, looking at the energetic loss (sterilization and  
526 lower methane yield) and the additional economic cost (mycelium and labor) this strategy will not be  
527 profitable at full scale. Finally, sterilization process, consisting of a thermo-alkaline post-treatment,  
528 showed an increase in energy recovery from SD of 13%. Further optimization of this post-treatment  
529 may enhance efficiency of SD recirculation strategy to offset plant methane production losses.

### 530 **Acknowledgment**

531 National Research and Technology Association (ANRT) is gratefully acknowledged for the PhD grant  
532 allocated to Ulysse Brémond (reference CIFRE N° 2016/0617). Jean-Michel Savoie from INRA MycSA  
533 is also gratefully acknowledged for his preliminary advice on this research.

### 534 **Declaration of interest**

535 Declarations of interest: none

### 536 **References**

- 537 [1] A. Sikder, J. Inekwe, M. Bhattacharya, Economic output in the era of changing energy-mix for  
538 G20 countries: New evidence with trade openness and research and development  
539 investment, *Appl. Energy*. 235 (2019) 930–938. doi:10.1016/j.apenergy.2018.10.092.
- 540 [2] R. Lora Grando, A.M. de Souza Antune, F.V. da Fonseca, A. Sánchez, R. Barrena, X. Font,  
541 Technology overview of biogas production in anaerobic digestion plants: A European  
542 evaluation of research and development, *Renew. Sustain. Energy Rev.* 80 (2017) 44–53.  
543 doi:10.1016/j.rser.2017.05.079.
- 544 [3] N. Scarlat, J. Dallemand, F. Fahl, Biogas: Developments and perspectives in Europe, *Renew.*  
545 *Energy*. 129 (2018) 457–472. doi:10.1016/j.renene.2018.03.006.
- 546 [4] European Biogas Association, EBA Statistical Report 2017, 2017. [http://european-  
547 biogas.eu/2017/12/14/eba-statistical-report-2017-published-soon/](http://european-biogas.eu/2017/12/14/eba-statistical-report-2017-published-soon/).
- 548 [5] W.M. Budzianowski, A review of potential innovations for production, conditioning and  
549 utilization of biogas with multiple-criteria assessment, *Renew. Sustain. Energy Rev.* 54 (2016)  
550 1148–1171. doi:10.1016/j.rser.2015.10.054.
- 551 [6] K. Hagos, J. Zong, D. Li, C. Liu, X. Lu, Anaerobic co-digestion process for biogas production:  
552 Progress, challenges and perspectives, *Renew. Sustain. Energy Rev.* 76 (2017) 1485–1496.  
553 doi:10.1016/j.rser.2016.11.184.
- 554 [7] Q. Zhang, J. Hu, D. Lee, Biogas from anaerobic digestion processes: Research updates, *Renew.*  
555 *Energy*. 98 (2016) 108–119. doi:10.1016/j.renene.2016.02.029.
- 556 [8] I. Muha, B. Linke, G. Wittum, A dynamic model for calculating methane emissions from  
557 digestate based on co-digestion of animal manure and biogas crops in full scale German  
558 biogas plants, *Bioresour. Technol.* 178 (2015) 350–358. doi:10.1016/j.biortech.2014.08.060.

- 559 [9] I. Angelidaki, K. Boe, L. Ellegaard, Effect of operating conditions and reactor configuration on  
560 efficiency of full-scale biogas plants, *Water Sci. Technol.* 52 (2005) 189–194.  
561 doi:10.2166/wst.2005.0516.
- 562 [10] L. Maldaner, C. Wagner-riddle, A.C. Vanderzaag, R. Gordon, C. Duke, Methane emissions from  
563 storage of digestate at a dairy manure biogas facility, *Agric. For. Meteorol.* 258 (2018) 96–107.  
564 doi:10.1016/j.agrformet.2017.12.184.
- 565 [11] J. Bacenetti, C. Sala, A. Fusi, M. Fiala, Agricultural anaerobic digestion plants: What LCA  
566 studies pointed out and what can be done to make them more environmentally sustainable,  
567 *Appl. Energy.* 179 (2016) 669–686. doi:10.1016/j.apenergy.2016.07.029.
- 568 [12] L. Lijó, Y. Lorenzo-toja, S. González-garcía, J. Bacenetti, M. Negri, M. Teresa, Eco-efficiency  
569 assessment of farm-scaled biogas plants, *Bioresour. Technol.* 237 (2017) 146–155.  
570 doi:10.1016/j.biortech.2017.01.055.
- 571 [13] F. Monlau, C. Sambusiti, E. Ficara, A. Aboulkas, A. Barakat, H. Carrère, New opportunities for  
572 agricultural digestate valorization: Current situation and perspectives, *Energy Environ. Sci.* 8  
573 (2015) 2600–2621. doi:10.1039/c5ee01633a.
- 574 [14] C. Sambusiti, F. Monlau, E. Ficara, A. Musatti, M. Rollini, A. Barakat, F. Malpei, Comparison of  
575 various post-treatments for recovering methane from agricultural digestate, *Fuel Process.*  
576 *Technol.* 137 (2015) 359–365. doi:10.1016/j.fuproc.2015.04.028.
- 577 [15] U. Brémond, R. De Buyer, J. Steyer, N. Bernet, H. Carrere, Biological pretreatments of biomass  
578 for improving biogas production : an overview from lab scale to full-scale, *Renew. Sustain.*  
579 *Energy Rev.* 90 (2018) 583–604. doi:10.1016/j.rser.2018.03.103.
- 580 [16] A. Musatti, E. Ficara, C. Mapelli, C. Sambusiti, M. Rollini, Use of solid digestate for  
581 lignocellulolytic enzymes production through submerged fungal fermentation, *J. Environ.*  
582 *Manage.* 199 (2017) 1–6. doi:10.1016/j.jenvman.2017.05.022.
- 583 [17] L. Mejias, A. Cerda, R. Barrena, T. Gea, Microbial Strategies for Cellulase and Xylanase  
584 Production through Solid-State Fermentation of Digestate from Biowaste, *Sustainability.* 10  
585 (2018) 2433. doi:10.3390/su10072433.
- 586 [18] W. Fang, P. Zhang, X. Zhang, X. Zhu, J.B. Van Lier, H. Spanjers, White rot fungi pretreatment to  
587 advance volatile fatty acid production from solid-state fermentation of solid digestate:  
588 Efficiency and mechanisms, *Energy.* 162 (2018) 534–541. doi:10.1016/j.energy.2018.08.082.
- 589 [19] J. Zhou, S. Song, Z. Huang, L. Yang, A. Jiao, Y. Liu, S. Wang, Cultivation of *Pleurotus Ostreatus*, a  
590 potential candidate for biogas residues degradation, *BioResources.* 13 (2018) 5432–5449.
- 591 [20] Ć. Jasmina, M. Gali, J. Vukojevi, I. Br, M. Staji, Potential of selected fungal species to degrade  
592 wheat straw, the most abundant plant raw material in Europe, *BMC Plant Biol.* 17 (2017) 249.  
593 doi:10.1186/s12870-017-1196-y.
- 594 [21] G. Chiavari, O. Francioso, G.C. Galletti, R. Piccaglia, F. Zadrazil, Characterization by pyrolysis-  
595 gas chromatography of wheat straw fermented with white rot fungus *Stropharia*  
596 *Rugosoannulata*, *J. Anal. Appl. Pyrolysis.* 15 (1989) 129–136.
- 597 [22] D.A. Wood, G.F. Leatham, Lignocellulose degradation during the life cycle of *Agaricus*  
598 *bisporus*, *FEMS Microbiol. Lett.* 20 (1983) 421–424.
- 599 [23] F. Guilayn, J. Jimenez, M. Rouez, M. Crest, D. Patureau, Digestate mechanical separation:

- 600 Efficiency profiles based on anaerobic digestion feedstock and equipment choice, *Bioresour.*  
601 *Technol.* 274 (2019) 180–189. doi:10.1016/j.biortech.2018.11.090.
- 602 [24] D. Wang, A. Sakoda, M. Suzuki, Biological efficiency and nutritional value of *Pleurotus*  
603 *ostreatus* cultivated on spent beer grain, *Bioresour. Technol.* 78 (2001) 293–300.
- 604 [25] C. Sánchez, Lignocellulosic residues: Biodegradation and bioconversion by fungi, *Biotechnol.*  
605 *Adv.* 27 (2009) 185–194. doi:10.1016/j.biotechadv.2008.11.001.
- 606 [26] APHA, Standard methods for the examination of water and wastewater. American Public  
607 Health Association, Washington, DC., Am. Public Heal. Assoc. Washington, DC. (2005).
- 608 [27] F. Braun, J. Hamelin, G. Gévaudan, D. Patureau, Development and application of an enzymatic  
609 and cell flotation treatment for the recovery of viable microbial cells from environmental  
610 matrices such as anaerobic sludge, *Appl. Environ. Microbiol.* 77 (2011) 8487–8493.  
611 doi:10.1128/AEM.05549-11.
- 612 [28] F. Monlau, A. Barakat, J.P. Steyer, H. Carrere, Comparison of seven types of thermo-chemical  
613 pretreatments on the structural features and anaerobic digestion of sunflower stalks,  
614 *Bioresour. Technol.* 120 (2012) 241–247. doi:10.1016/j.biortech.2012.06.040.
- 615 [29] C. Holliger, M. Alves, D. Andrade, I. Angelidaki, S. Astals, U. Baier, C. Bougrier, P. Buf, M.  
616 Carballa, V. De Wilde, F. Ebertseder, B. Fernández, E. Ficara, I. Fotidis, J. Frigon, H.F. De Laclos,  
617 S.M. Ghasimi, G. Hack, M. Hartel, J. Heerenklage, I.S. Horvath, P. Jenicek, K. Koch, J.  
618 Krautwald, J. Lizasoain, J. Liu, L. Mosberger, M. Nistor, H. Oechsner, J.V. Oliveira, M. Paterson,  
619 A. Pauss, S. Pommier, I. Porqueddu, F. Raposo, T. Ribeiro, F.R. Pfund, S. Strömberg, M.  
620 Torrijos, G. Hack, D. Andrade, C. Bougrier, K. Koch, Towards a standardization of biomethane  
621 potential tests, *Water Sci. Technol.* 74 (2016) 2515–2522. doi:10.2166/wst.2016.336.
- 622 [30] J. Jimenez, Q. Aemig, N. Doussiet, J. Steyer, S. Houot, D. Patureau, A new organic matter  
623 fractionation methodology for organic wastes : Bioaccessibility and complexity  
624 characterization for treatment optimization, *Bioresour. Technol.* 194 (2015) 344–353.  
625 doi:10.1016/j.biortech.2015.07.037.
- 626 [31] ISO 13906, Animal Feeding Stuffs: Determination of Acid Detergent Fibre (ADF) and Acid  
627 Detergent Lignin (ADL) Contents, International Organization for Standardization, Geneva,  
628 Switzerland, 2008.
- 629 [32] a. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton, D. Crocker, NREL/TP-510-  
630 42618 analytical procedure - Determination of structural carbohydrates and lignin in Biomass,  
631 *Lab. Anal. Proced.* (2012) 17. doi:NREL/TP-510-42618.
- 632 [33] C. Holliger, H. Fruteau de Laclos, G. Hack, Methane Production of Full-Scale Anaerobic  
633 Digestion Plants Calculated from Substrate's Biomethane Potentials Compares Well with the  
634 One Measured On-Site, *Front. Energy Res.* 5 (2017) 1–9. doi:10.3389/fenrg.2017.00012.
- 635 [34] M.B. Bellettini, F.A. Fiorda, H.A. Maievas, G.L. Teixeira, A. Suelen, P.S. Hornung, A.M. Junior,  
636 R.H. Ribani, Factors affecting mushroom *Pleurotus* spp., *Saudi J. Biol. Sci.* (2016).  
637 doi:10.1016/j.sjbs.2016.12.005.
- 638 [35] D. Hernandez, J.E. Sanchez, K. Yamasaki, A simple procedure for preparing substrate for  
639 *Pleurotus ostreatus* cultivation, *Bioresour. Technol.* 90 (2003) 145–150. doi:10.1016/S0960-  
640 8524(03)00118-4.
- 641 [36] D. Knop, O. Yarden, Y. Hadar, The ligninolytic peroxidases in the genus *Pleurotus*: divergence

- 642 in activities, expression, and potential applications, *Appl. Microbiol. Biotechnol.* 99 (2015)  
643 1025–1038. doi:10.1007/s00253-014-6256-8.
- 644 [37] H. Válková, Č. Novotný, K. Malachová, P. Šlosarčíková, J. Fojtík, Effect of bacteria on the  
645 degradation ability of *Pleurotus ostreatus*, *Sci. Total Environ.* 585 (2017) 1114–1120.  
646 doi:10.1016/j.scitotenv.2017.01.171.
- 647 [38] X. Raynaud, N. Nunan, Spatial Ecology of Bacteria at the Microscale in Soil, *PLoS One.* 9 (2014).  
648 doi:10.1371/journal.pone.0087217.
- 649 [39] J. Tian, A. Pourcher, A. Bize, A. Wazeri, P. Peu, Impact of wet aerobic pretreatments on  
650 cellulose accessibility and bacterial communities in rape straw, *Bioresour. Technol.* 237 (2017)  
651 31–38. doi:10.1016/j.biortech.2017.03.142.
- 652 [40] O. Romero-arenas, M.A.D. Huato, I.H. Treviño, J.F.C.P. Lezama, A.A. García, A.D.V. Arellano,  
653 Effect of pH on growth of the mycelium of *Trichoderma viride* and *Pleurotus ostreatus* in solid  
654 cultivation mediums, *African J. Agric. Res.* 7 (2012) 4724–4730. doi:10.5897/AJAR12.568.
- 655 [41] J.L. Ramos-suárez, D. Gómez, L. Regueiro, A. Baeza, F. Hansen, Alkaline and oxidative  
656 pretreatments for the anaerobic digestion of cow manure and maize straw: Factors  
657 influencing the process and preliminary economic viability of an industrial application,  
658 *Bioresour. Technol.* 241 (2017) 10–20. doi:10.1016/j.biortech.2017.05.054.
- 659 [42] E. Rouches, R. Escudié, E. Latrille, H. Carrère, Solid-state anaerobic digestion of wheat straw:  
660 Impact of S/I ratio and pilot-scale fungal pretreatment, *Waste Manag.* 85 (2019) 464–476.  
661 doi:10.1016/j.wasman.2019.01.006.
- 662 [43] J. Lalak, A. Kasprzycka, D. Martyniak, J. Tys, Effect of biological pretreatment of *Agropyron*  
663 *elongatum* “BAMAR” on biogas production by anaerobic digestion, *Bioresour. Technol.* 200  
664 (2016) 194–200. doi:10.1016/j.biortech.2015.10.022.
- 665 [44] A.M. Mustafa, T.G. Poulsen, K. Sheng, Fungal pretreatment of rice straw with *Pleurotus*  
666 *ostreatus* and *Trichoderma reesei* to enhance methane production under solid-state  
667 anaerobic digestion, *Appl. Energy.* 180 (2016) 661–671. doi:10.1016/j.apenergy.2016.07.135.
- 668 [45] A.M. Mustafa, H. Li, A.A. Radwan, K. Sheng, X. Chen, Effect of hydrothermal and Ca(OH)<sub>2</sub>  
669 pretreatments on anaerobic digestion of sugarcane bagasse for biogas production, *Bioresour.*  
670 *Technol.* 259 (2018) 54–60. doi:10.1016/j.biortech.2018.03.028.
- 671 [46] E. Rouches, S. Zhou, J.P. Steyer, H. Carrere, White-Rot Fungi pretreatment of lignocellulosic  
672 biomass for anaerobic digestion: Impact of glucose supplementation, *Process Biochem.* 51  
673 (2015) 1784–1792. doi:10.1016/j.procbio.2016.02.003.
- 674 [47] S.T. Chang, W.A. Hayes, *The biology and cultivation of edible mushrooms*, 1978.
- 675 [48] G. Maynaud, C. Druilhe, M. Daumoin, J. Jimenez, D. Patureau, M. Torrijos, A.M. Pourcher, N.  
676 Wéry, Characterisation of the biodegradability of post-treated digestates via the chemical  
677 accessibility and complexity of organic matter, *Bioresour. Technol.* 231 (2017) 65–74.  
678 doi:10.1016/j.biortech.2017.01.057.
- 679 [49] J. Du, Y. Qian, Y. Xi, X. Lü, Hydrothermal and alkaline thermal pretreatment at mild  
680 temperature in solid state for physicochemical properties and biogas production from  
681 anaerobic digestion of rice straw, *Renew. Energy.* 139 (2019) 261–267.  
682 doi:10.1016/j.renene.2019.01.097.

- 683 [50] J. Jimenez, H. Lei, J.P. Steyer, S. Houot, D. Patureau, Methane production and fertilizing value  
684 of organic waste: Organic matter characterization for a better prediction of valorization  
685 pathways, *Bioresour. Technol.* 241 (2017) 1012–1021. doi:10.1016/j.biortech.2017.05.176.
- 686 [51] N. Das, M. Mukherjee, Cultivation of *Pleurotus ostreatus* on weed plants, *Bioresour. Technol.*  
687 98 (2007) 2723–2726. doi:10.1016/j.biortech.2006.09.061.
- 688 [52] F. Zadrazil, A.K. Puniya, Influence of Carbon Dioxide on Lignin Degradation and Digestibility of  
689 Lignocellulosics Treated with *Pleurotus sajor-caju*, *Int. Biodeterior. Biodegradation.* (1994)  
690 237–244.
- 691