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# Solid-State Anaerobic Digestion of wheat straw: impact of S/I ratio and pilot-scale fungal pretreatment

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## Abstract

Solid State Anaerobic Digestion (SSAD) of fungal pretreated wheat straw was evaluated in a leach bed reactor. During a first experiment, the effect of Substrate/Inoculum (S/I) ratios on the start-up phase was investigated. High S/I increased methane productivity but also raised the risk of reactor failure due to Volatile Fatty Acid (VFA) accumulation. With S/I ratios between 1.2 and 3.6 (Volatile Solid (VS) basis), the SSAD start-up using wheat straw was successful. Moreover, reactors were able to recover from acidification when the Total VFA/alkalinity ratio was lower than 2 gHAc_eq/g$CaCO_3$, with VFA concentrations lower than 10 g/L and a pH close to 5.5. The conventional threshold of 0.6 gHAc_eq/g$CaCO_3$ for stable wet AD is therefore not adapted to SSAD.

During a second experiment, after the wheat straw was submitted to a fungal pretreatment in a non-sterile pilot-scale reactor, it was digested with an S/I ratio of 2.8/2.9. Under batch SSAD conditions, the biodegradability of pretreated wheat straw was slightly improved in comparison to the control (254 versus 215 NmL/g VS, respectively). Considering mass losses occurring during the pretreatment step, suboptimal pretreatment conditions caused a slightly lower methane



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production (161 *versus* 171 NmL/g $TS_{initial}$ after 60-days anaerobic digestion). Nevertheless, pretreatment improved the start-up phase with lower acidification relative to controls. It would be particularly beneficial to improve the methane production in reactors with short reaction times.

**Keywords**

Dry anaerobic digestion; white-rot fungi; lignocellulose; alkalinity; substrate/inoculum ratio

**Abbreviations**

AD: Anaerobic Digestion

A.F-D: autoclaved and freeze-dried

BRFM: Banque de Ressources Fongiques de Marseille, Bank of Fungal Ressources of Marseille

cMWS: fungal colonized Miscanthus and Wheat Straw, and the corresponding batch reactor

Diam: diameter

FWS: Fungal pretreated Wheat Straw and the corresponding batch reactor

HAc_eq: acetic acid equivalent

I: Inoculum

$I_L$: Liquid inoculum

$I_S$: Solid inoculum

LBR: Leach Bed Reactor

MC: Moisture Content

MWS: Miscanthus pellets and Wheat Straw, and the corresponding batch reactor

S: Substrate

S/I: Substrate/Inoculum

SSAD: Solid-State Anaerobic Digestion

TOC: Total Organic Carbon

TS: Total Solids

TVFA: Sum of Volatile Fatty Acids expressed in acetic acid equivalents

VFA: Volatile Fatty Acids

VS: Volatile Solids

WRF: White-Rot Fungi

## 1 Introduction

Anaerobic digestion (AD) is particularly noteworthy when several current challenges are addressed, such as the depletion of fossil resources, the fight against global warming or the reduction of waste. This process involves the bioconversion of organic matter into biogas (mainly $CO_2$ and $CH_4$). The resulting residue (called digestate) can often be valorised as a fertilizer for agriculture, while methane provides a source of energy which can be converted into electricity, heat or biofuel (Ge et al., 2016).

In Europe, a large part of the AD treatment capacity for solid waste involves Solid-State Anaerobic Digestion (SSAD) (De Baere, 2000). SSAD processes are often characterized by a Total Solid (TS) content greater than 15%, even though the term semi-dry anaerobic digestion is generally used for a TS content between 15 and 20% (Li et al., 2011; Motte et al., 2013). SSAD processes are less costly (especially batch processes), they require smaller and simpler reactor designs (fewer moving parts, lower energy requirement for heating) and digestate management is

110 easier (absence of phase separation) (Li et al., 2011). Moreover, SSAD is especially adapted for
111 lignocellulosic substrates (Ge et al., 2016) as their moisture content is low.
112 Cereal residues, such as straw, represent an interesting substrate for anaerobic digestion because
113 they are rich in carbohydrates and widely available with a worldwide annual production of more
114 than $6x10^9$ Mg (Gabrielle and Gagnaire, 2008). In comparison to other energy recovery processes
115 such as incineration, anaerobic digestion of straw presents the advantage of preserving some
116 carbon in the digestate, making possible its return to soil. Indeed, soil organic matter is essential
117 for soil fertility and straw-to-energy chain sustainability (Gabrielle and Gagnaire, 2008).
118 SSAD in batch leach bed reactors represents an adequate process for straw digestion (Andre et
119 al., 2018; Karthikeyan and Visvanathan, 2013). During such a dry process, the liquid phase is
120 sprinkled over the solid phase composed of substrate and inoculum, which are loaded into the
121 reactor. Batch processes require less capital costs and are relatively simpler to operate (Li et al.,
122 2011). For solid substrate with low degradability and/or a C/N ratio greater than 15, single-stage
123 processes (all anaerobic digestion steps occur in a single reactor) are easier to operate (Kusch et
124 al., 2008; Weiland, 1993). Leachate recycling favours homogenization which in turn facilitates
125 the complete degradation of the substrate (Brummeler et al., 1992). Continuous watering
126 increases the risk of spreading acidification during process initiation whereas discontinuous
127 leachate recycling is rather assumed to expand methanogenic areas (Kusch et al., 2012).
128 Straws contain high amounts of soluble compounds (12% TS for wheat straw (Sun, 2010)) that
129 can be rapidly converted into VFA (Volatile Fatty Acids). If the amount of methanogens in the
130 inoculum is insufficient, VFA accumulation can occur because of lower growth rate for
131 methanogens compared to acidogenic bacteria (Vavilin and Angelidaki, 2005). This
132 accumulation can lead to a detrimental pH drop inducing a process failure (inhibition or death of
133 methanogens), especially during a batch start-up phase (Brown and Li, 2013). The optimum pH

for methanogen archaea lies between 7 and 8 even though anaerobic digestion can occur between 6 and 8.3 (Angelidaki and Sanders, 2004). Nevertheless, high substrate loadings favour higher methane productivity (production per reactor volume) but also increase the risk of acidification. Thus, in order to avoid this risk during the start-up phase, the optimum S/I (Substrate/Inoculum) ratio for the SSAD of a given substrate is a key parameter to assess (Kusch et al., 2011). It implies an efficient monitoring that remains a real challenge in plants (Charnier et al., 2016). Finally, lignocellulosic biomass contains lignin that is poorly biodegradable during anaerobic digestion. Lignin also restricts access to fermentable sugars for hydrolytic bacteria and enzymes and thus impedes methane production during anaerobic digestion. Pretreatments are therefore necessary to disrupt the lignin matrix, with a further objective to improve the hydrolysis rate during anaerobic digestion. Biological pretreatments (enzymes, fungi…) are generally more environmentally friendly and cheaper than other existing processes such as grinding, steam explosion or chemical pretreatments using corrosive reagents. Among the biological techniques, White-Rot Fungi (WRF), degrading wood in nature, have proven to be an economical and efficient way to delignify a substrate and to increase its methane production (Rouches et al., 2016a). Although studies on WRF pretreatment for anaerobic digestion receive increasing interest, there is still a need for further knowledge (Rouches et al., 2016a), especially concerning its industrial feasibility. The almost systematic application of sterile processes for fungal pretreatment would not be feasible on biogas plants due to excessive additional costs (Zhao et al., 2014). Previous work has demonstrated the significance of WRF, *Polyporus brumalis* BRFM 985 (Banque de Ressources Fongiques de Marseille) for pretreating wheat straw before wet anaerobic digestion (Rouches et al., 2016b). In contrast, the evaluation of fungal pretreated lignocellulose SSAD is still very scarce in the literature.

The objective of this study was to maximise methane productivity from SSAD of wheat straw in leach bed reactor by determining adequate S/I ratio and fungal pretreatment of the substrate. A first experiment using different S/I ratios allowed investigating acidification risk during the start-up phase and the reactor recovery capacity following an acidification period. Based on previous S/I determinations, a second experiment was carried out to investigate the SSAD of wheat straw pretreated with *P. brumalis* BRFM 985 by solid-state fermentation in an unsterile pilot reactor.

# 2 Material and methods

## 2.1 Wheat straw

Winter wheat straw (*Triticum aestivum*), harvested in the North of France in 2012, was collected from bales stored in a sheltered area. As reported previously (Rouches et al., 2018), the NREL composition was 37.5 % TS cellulose, 27.5 % TS hemicelluloses and 23.0 % TS lignin. The straw was autoclaved and freeze-dried (A.F-D) only for Experiment II as those steps were required for the fungal pretreatment. Consequently, no differences between Experiment II reactors would be due to an influence of those operations on hydrodynamics.

## 2.2 Solid State Anaerobic Digestion

### 2.2.1 SSAD Leach Bed Reactors (LBR) design

Similarly to Riggio et al. (2017), experiments were performed in batch mode using four 6-L glass reactors (head-space ≈ 1 L, leachate tank ≈ 1.5 L), equipped with water jacket maintaining temperature at 37°C (Fig.1). Leachate was collected into a liquid-phase reservoir at the base of reactors. The solid phase was not submerged by the liquid since the whole liquid phase was

contained in the liquid reservoir. Peristaltic pumps were set with timers (5 min) to sprinkle the whole leachate volume over the biomass bed every 2 hours. Biogas production was continuously measured using a flowmeter (milligas counter-1V3.0 PMMA, Ritter Inc., Germany). The gas flow rate was acquired every 2 min by a computer.

Fig.1. Schematic diagram of laboratory-scale SSAD digesters (6 L). The inoculum/substrate mix is separated from the leachate tank with a sieve and the leachate is discontinuously recycled.

### 2.2.2 Monitoring of SSAD chemical parameters

VFA and pH on leachate samples were measured regularly according to the extent of their variations (daily at the beginning, weekly at the end).

Only a few alkalinity measurements were performed in the leachate, with pH titration using 0.1M HCl to an endpoint of 4.3 (Ripley et al., 1986). These were expressed in g equivalent $CaCO_3$/L.

The first measurement occurred on day 1 rather than day 0, to favour the homogeneity of the mixture (moisture content, temperature).

After pH-measurement (calibrated pH-meter EUTECH Instrument®, pH 510), a centrifugation step with a micro-spin (20 min at 13 400 rpm) was made. Then, supernatant was diluted with an equal volume of internal standard as a reference for calculation of the VFA concentrations of the solutions. VFA concentrations were quantified by gas chromatography using a Clarus GC 580 (PerkinElmer, USA) equipped with an auto-sampler and coupled to flame ionization detection (250°C) with $H_2$ and air as burning gas. Injector temperature was 220°C. Elite FFAP (PerkinElmer, USA) column (15 m long, 0.53 mm i.d., 1 µm thickness) was used with nitrogen (Nitrogen gas 5.0) as carrier gas at a flow of 7 mL/min. The GC oven temperature was programed

to increase from 80 to 120°C (hold time 6.5 min) and from 120°C to 140°C (hold time 3 min). Acetic acid equivalents of VFA were used to calculate the TVFA/alkalinity ratio: 1.0 ($C_2$), 0.818 ($C_3$), 0.682 ($C_4$), 0.588 ($C_5$) and 0.515 ($C_6$) (Raposo et al., 2006).

Biogas composition in the head space was measured at the same frequency as for leachate sampling. It was measured with a Clarus GC 480 (PerkinElmer, USA) equipped with two columns maintained at 65°C: the first (RtUbond) served to separate $O_2$, $N_2$, $CH_4$, while second (RtMolsieve) served to separate $H_2S$ and $CO_2$ from other gases. The carrier gas was helium at 50 $mL.min^{-1}$ and with a pressure of 36 psi. The injector and detector temperature was 200°C. Gaseous compounds were detected using a thermal conductivity detector. Calibration was ensured with a standard gas composed of 0.1% $H_2S$, 0.5% $O_2$, 10% $N_2$, 25% $CO_2$, and 64.4% $CH_4$. Methane volumes are expressed in standard temperature and pressure conditions (NmL) after subtracting the endogenous methane production.

### 2.3 Experiment I: influence of S/I ratio on SSAD

To investigate the risk of acidification, four reactors were launched simultaneously with different amounts of substrate and inoculum. The substrate was wheat straw whose total solid (TS) and volatile solid (VS) contents are reported in Table 1. TS (48h at 105°C) and VS (3h at 550°C) were measured according to Standard Methods (APHA, 1998). The inoculum (liquid and solid inoculum) was sampled from a stable full-scale LBR operated at 42°C. Feedstock was cow manure (i.e. containing a high proportion of straw) and SSAD lasted 42 days. To exhaust biodegradable material and reduce endogenous methane production, 1.7 kg of the solid digestate were left at 37°C with 1.5 L of tap water in 6-L batch reactors in duplicate for one month before measuring its TS and VS contents (Table 1) and further using as inoculum in LBR. The amounts

of substrate, solid and liquid inocula are reported in Table 2. S/I ratios (VS basis) were: 1.2, 2.0, 3.6 and 8.5 with a major proportion of solid inoculum which represented from 76 to 94% of total inoculum.

Contrary to full scale plants, not enough liquid inoculum was available to start the batches and it was chosen to add a slightly buffered solution. Indeed, the total liquid (tap water plus liquid inoculum) was buffered with $NaHCO_3$ to the medium concentration of 1.3 g/L (half of the concentration used for BMP tests), (Rouches et al., 2016b). This method allowed to obtain recommended TVFA/alkalinity ratios (<0.4, as further explained) from the beginning of anaerobic digestion (day 1, Table 3).

Table 1. Characteristics of substrates and inocula used in Experiments I and II. TS and VS content and total nitrogen concentrations.

Table 2. Experimental set-up for Experiments I and II.

Wheat straw and solid inoculum were hand-mixed in a bag, transferred to a reactor and pressed for one minute with an 8 kg weight to imitate the compaction effect that takes place in plants (Riggio et al., 2017). The TS content varied between 15 and 17% within reactors (Table 2). Head spaces were flushed with nitrogen gas. Measurements ended after 34 days (for S/I=8.5) and after the VFA peak for other S/I ratios.

## 2.4 Experiment II: effect of fungal pretreatment on SSAD

### 2.4.1 Fungal inoculum

The *Polyporus brumalis* BRFM 985 strain was provided by the "Centre International de Ressources Microbiennes" (CIRM-CF).

*P. brumalis* BRFM 985 was first cultivated on liquid medium (malt extract broth 20 g/L in Roux flasks) which were inoculated with five 5-mm diameter agar discs of 7-day-old mycelia grown on MA2 (malt extract broth 20 g/L and agar 20 g/L). The Roux flasks were closed with cotton plugs and incubated for seven days at 30°C. The mycelium of the liquid culture was harvested, mixed with 25 mL sterile mQ water and ground for one minute using a hand blender. 10 mL of crushed mycelium were mixed with 40 mL sterile mQ water. This fungal suspension was used for inoculating 50 g autoclaved miscanthus Terr'nova® pellets (48% cellulose, 27% hemicelluloses, 24% lignin). Miscanthus pellets were incubated in Roux flasks for 24 h at 30°C before adding the 25 mL sterile mQ water. Culture on miscanthus pellets lasted between 7 and 10 days. To favour colonization, the flasks were shaken manually each day. Finally, the fungal inoculated miscanthus pellets were employed as solid inoculum for wheat straw pretreatment. The use of such a support for inoculum is supposed to enhance fungal growth and colonization capacity (Rama et al., 2001). All materials and culture medium to obtain fungal inoculum were sterile (autoclaved for 20 min at 120°C).

### 2.4.2 Fungal pretreatment of wheat straw

One Roux flask containing fungal colonized miscanthus pellets was used to inoculate 200 g of sterile straw (Fig. 2A). The straw was autoclaved in a bag holding about 113 mL of mQ water/100 g straw. 255 mL sterile mQ water/100 g straw and 5 mL/100 g straw of metal solution ($CuSO_4$ and $FeSO_4$ at 18 mmol/L) were then filtered at 0.2 µm and added to the straw under

sterile conditions. The bags were manually shaken to ensure a good distribution of the fungal inoculum. Finally, the seeded straw was placed into a 40 L aerated reactor (Fig. 1B) under clean conditions. Before fungal inoculation, the reactor was cleaned with a Kärcher pressure washer for 20 minutes at 120°C. The aerobic reactor was equipped with two trays: each one received 200 g of straw cut with scissors (1-5 cm). Fungal cultivation on straw lasted 13 days in the aerated reactor under a high moisture content (≈90%) and 31°C. Pretreated straw was freeze-dried before further utilization.

Fig. 2. Schematic representation of the fungal pretreatment. A. Pretreatment inputs. B. Aerobic pilot-reactor design for wheat straw pretreatment with *Polyporus brumalis*, BRFM 985. Moist air travels through the thermostatted pretreatment reactor equipped with two trays containing straw and fungal inoculum. Diam: diameter; MC: Moisture Content. C. Picture of pretreated wheat straw used in Experiment II. White areas correspond to the mycelium of *P. brumalis* BRFM 985.

### 2.4.3 SSAD of fungal pretreated wheat straw

To have a fresh inoculum adapted to the substrate with low endogenous methane production; the solid anaerobic inoculum consisted of a mixture of reactor digestates and of a part of starved inoculum prepared as in Experiment I. The digestates resulted from Experiment I with S/I ratios of 1.2 and 2.0 after two months of SSAD. The liquid inoculum was made of a mixture of leachates recovered at the end of Experiment I (which did not contain any VFA) and of tap water buffered with $NaHCO_3$ to the concentration of 1.3 g/L. The first three reactors served to investigate the SSAD of pretreated wheat straw while the fourth one was dedicated to the

290 measurement of inoculum endogenous methane production. The latter only contained inoculum
291 and leachate (Table 2). A first reactor (reactor FWS) was fed with freeze-dried fungal-pretreated
292 straw from the pilot-reactor (see 2.4.2). As the pretreated straw (Fig. 2) contained colonized
293 miscanthus pellets (used as fungal inoculum), the other two reactors were reserved for measuring
294 the influence of miscanthus pellets on SSAD. One reactor (reactor MWS) was fed with A. F-D
295 straw and miscanthus pellets (autoclaved and freeze-dried). The second one (reactor cMWS) was
296 fed with A. F-D straw and *Polyporus brumalis* BRFM 985-colonized miscanthus pellets (Table
297 1) prepared as in 2.4.1. The amount of miscanthus per reactor was representative of the
298 proportion used for straw pretreatment. Reactors MWS and cMWS were used as controls. The
299 methane production due to the presence of miscanthus in pretreated straw could be determined
300 with reactor MWS, while the methane production resulting from the fungal inoculum used to
301 pretreat the wheat straw was taken into account with reactor cMWS. The impact of straw fungal
302 modifications on methane production could be assessed by comparing their results with reactor
303 FWS.
304 The TS and VS of the different substrates and inocula are reported in Table 1. The TS contents
305 and amounts of VS were similar for all three cMWS, MWS and FWS reactors (Table 2). S/I
306 ratios were 2.8 or 2.9 with solid inoculum accounting for 88% of total inoculum.
307

308 **2.4.4 Analysis of the final leachate and digestate composition**

309 Solid digestates were freeze-dried and milled using bill milling before Total Organic Carbon
310 (TOC) and Total Kjedahl Nitrogen (TKN) analysis whereas leachates were filtered through a
311 0.54 µm pore size screen.

#### 2.4.4.1 Total Organic Carbon (TOC)

TOC was measured in duplicates with a carbon analyser (TOC-V CSN, Shimadzu and solid sample module-5000A). The sample is burnt at 900°C with a cobalt/platinum catalyst and pure oxygen, released carbon dioxide is measured by a non-dispersive infrared detector. Glucose was used as control.

#### 2.4.4.2 Total Kjeldahl Nitrogen (TKN)

Kjeldahl nitrogen (TKN) was titrated in duplicates using a Buchi 370-K distillater/titrator after mineralization of samples with a Buchi digestion unit K438.

#### 2.4.4.3 Ammonium concentration in final leachate

Ammonium ($NH_4^+$) concentrations were measured in duplicates with an ion chromatography system (ICS 3000 Dionex, USA) equipped with two pre-columns (NG1-2mm and CG16-2mm) and a separation column CS16-3mm. After the eluate passed through a Cation Self-Regenerating Suppressor (CSRS-300-2mm), detection was carried out by conductivity. The eluent was hydroxymethanesulfonic acid (HMSA) with a concentration gradient ranging from 25 to 40 mM and a flow rate of 0.3 mL $min^{-1}$.

#### 2.4.4.4 Analysis of Variance (ANOVA)

Analysis of variance (ANOVA, $\alpha = 0.1$) was carried out using R software (version 3.2.1) with "lattice" and "lawstat" libraries. Multiple mean comparisons were performed with the Tukey HSD (Honest Significant Difference) test at the same significance threshold of 0.1. The chosen threshold was slightly higher than the common one of 0.05 due to the heterogeneity of the solid matrices and the small population available (n=2).

# 3 Results and discussion

## 3.1 Experiment I: effect of S/I ratio

### 3.1.1 Evaluation of the start-up phase

During Experiment I the performances of several Substrate/Inoculum ratios (S/I) were investigated at the reactor start-up (measurements were stopped after VFA peak). Fig. 3 presents the main results: pH and VFA in leachate and methane production. Only the start-up phase was studied since it is the most critical step when substrate overloading may lead to acidification.

Fig. 3. Main parameters of anaerobic digestion during Experiment I. Methane production (A), leachate pH (B) and leachate total VFA concentration (C) and daily methane production (D) in function of time for several S/I ratios.

Four S/I ratios were investigated: 1.2, 2.0, 3.6 and 8.5 (VS basis). For S/I ratios of 1.2 and 2.0, methane production occurred all along the duration of the experiment (Fig.3A), while the pH remained close to neutrality during the start-up phase (Fig.3B). Finally, a peak in daily methane production almost reached 8 NmL/g $VS_{tot}$/d (Fig.3D). These elements correspond to good performance reactors.

Nonetheless, the VFA peak (6.8 g/L) was higher for an S/I=2.0 than for an S/I=1.2 (5 g/L) due to the higher amount of substrate (Fig. 3C). For S/I=2.0, the lowest pH value (6.5) was observed at the VFA peak (≈day 5). This also coincided with a decrease in the daily methane production (Fig. 3D) leading to a delay in the main methane production peak (day 9). Indeed, the daily methane production peak occurred after the VFA peak (contrary to observations for the S/I=1.2 reactor) when the pH increased, thus pointing to the necessity for methanogens to adapt. This reactor was

357 close to instability due to a plateau phase in VFA (from day 4 to 8). Moreover, compared to
358 S/I=1.2, methane production at day 15 was slightly lower (≈ 10 NmL/g $VS_{total}$, Fig. 3A) whereas
359 it was similar until the minimum pH (day 4).
360 With S/I = 8.5, acidification occurred and the reactor never recovered until day 34 when it was
361 stopped. The pH never exceeded 5.5 after the third day (Fig.3B). Following day 3, the daily
362 methane production remained very low (Fig.3D), and the cumulated methane yield reached 5
363 NmL/g $VS_{total}$ on day 7 and never increased afterwards (Fig.3A). The total VFA reached a high
364 concentration of 10 g/L on day 6 and progressively increased until 11 g/L for the last
365 measurement (day 27) (Fig.3C), thus pointing out that they were not consumed by methanogens.
366 The small VFA production between day 6 and day 27 suggests that even hydrolytic and
367 acidogenic microorganisms were affected by the low pH conditions. The optimal pH range for
368 these microorganisms lies between 5.5 and 6.5 (Jha et al., 2011).
369 With S/I = 3.6, acidosis took place during almost ten days (pH ≈ 5.5 from day 5 to 15). The daily
370 methane production decreased and even halted on day 8. It then steadily increased after day 15
371 (Fig.3D) although never exceeding 5 NmL/g $VS_{total}$/d. During acidosis, a plateau was observed
372 for the methane yield (Fig.3A), reflecting the weak daily methane production as well as the
373 instability of anaerobic digestion. From day 6 to 10, the total VFA concentrations for S/I = 3.6
374 and 8.5 were similar (10 g/L) (Fig.3C) whereas a small difference in pH was observed (0.2 more
375 for S/I = 3.6). Despite similarities between certain parameters, one reactor recovered while
376 another failed. Indeed, for S/I = 3.6, a decrease in the VFA concentration occurred between day
377 20 and 30 concomitantly with an increase in pH and with a moderate methane production. The
378 pH was always higher for S/I = 3.6 than for S/I = 8.5 (Fig.3B). This phenomenon may be
379 explained by the difference in alkalinity between the two reactors on day 10: the S/I = 3.6
380 presented a 0.8 g $CaCO_3$/L higher concentration than the S/I=8.5 reactor, while the pH of S/I=3.6

reactor remained close to 5.5 (Table 3). As alkalinity is related to the buffer capacity of the medium, it needs to be sufficiently elevated. In a stable liquid reactor, alkalinity frequently varies between 2 and 4 $gCaCO_3/L$ (APHA, 1998). Alkalinity measurements in this study remained within this range but decreased sharply at day 10 for S/I=8.5 reactor (Table 3).

Table 3. Alkalinity, TVFA/alkalinity and pH on days 1 and 10 of the Experiment I. Parameters at day 10 for S/I=3.6 led to a recovery of methanogenic activity whereas for S/I= 8.5, inhibition persisted.

Methane yield with S/I =2.0 reached 97 NL/kg VS at day 15. Compared to other studies with S/I=2, this was slightly better than the 90 L $CH_4$/kg VS (final production at day 30) obtained by Cui et al. (2011) or the 66 L $CH_4$/kg VS obtained by Liew et al. (2012) with a 22% TS content. This particularly efficient methane production could possibly be due to a moderate TS content (Motte et al., 2013), to leachate recycling (Kusch et al., 2008) and to a better choice of inoculum origin: Cui et al. (2011) and Liew et al. (2012) used effluents from municipal solid waste as inoculum.

The current study is in agreement with the literature: for example, for an S/I between 2 and 4, a similar methane production with wheat straw was obtained in a 30-day batch SSAD at 22% TS without leachate recycling (Liew et al., 2012). With higher S/I ratios, a drastic fall in the methane yield was observed. Consequently, higher ratios, even under other operating conditions, do not seem to be adapted to batch mono-digestion of wheat straw.

### 3.1.2 TVFA/alkalinity as process stability indicator

Anaerobic reactor monitoring is of primary interest for the economical profitability of plants. As reported by Ahring et al. (1995), several authors have suggested monitoring VFA to evaluate reactor stability. However each reactor appears to have its own “normal” level of VFA.

The detrimental effect of high VFA concentrations could be mitigated thanks to the high buffer capacity of the reacting medium which is generally estimated by its alkalinity. Several studies have highlighted the ratio between VFA and alkalinity as an efficient parameter for controlling an anaerobic digestion plant.

The anaerobic digestion process is stable if the TVFA (Total VFA in g HAc eq/L)/alkalinity (g CaCO3/L) ratio remains between 0.3 and 0.4 (Lili et al., 2011; Lossie and Pütz, 2008; Raposo et al., 2006). While some authors consider 0.6 to be a critical threshold (Lossie and Pütz, 2008), others report a threshold of 0.8 (Callaghan et al., 2002; Kim and Kafle, 2010). However, this criterion may not be adapted to SSAD where inhibitor concentrations are high because of poor dilution (Rapport et al., 2008). The deficiency of moisture content in substrate also leads to mass transfer limitation, and particularly gas-liquid transfer necessary for the functioning of the anaerobic ecosystem (Abbassi-Guendouz et al., 2012) but biogas production per reactor volume can be comparable to wet digestion if SSAD conditions are optimal (Kusch et al., 2011).

Nonetheless, when using a continuously stirred liquid tank fed with Chinese cabbage silage and swine manure, reactors remained stable with a TVFA/alkalinity ratio close to 1 but a VFA below 10 g/L (Kim and Kafle, 2010). Even though the configuration of Experiment I was very different from this case, the limit of 10 g/L for VFA was also observed since, above this limit, the reactor failed (S/I = 8.5). Similarly, with wheat straw and an SSAD batch (22% TS), reactor failure was

425 also observed when the final VFA level reached 12.4 g/kg (Cui et al., 2011). However, Duan et
426 al. (2012) considered that, without a detrimental pH drop, VFA accumulation could enhance the
427 multiplication of methanogens.

428 The TVFA/alkalinity ratio appears to be a preferential monitoring parameter, although several
429 precautions should be taken to ensure a robust measurement (sample preparation, alkalinity and
430 VFA measurement methods, *etc*.). Adequate initial and final TVFA/alkalinity ratios are not
431 sufficient to indicate the absence of an acidification stage. This ratio should be monitored
432 dynamically during anaerobic digestion, as a high increase can reflect the potential instability of
433 the reaction (Kim and Kafle, 2010; Voß et al., 2009).

434 Finally, the evaluation of the process stability should be completed by the methane yield because
435 of the existence of an inhibited steady-state (Chen et al., 2008; Kim and Kafle, 2010). In
436 Experiment I, an inhibited steady-state is unlikely since, according to Kusch et al. (2011), 49% of
437 the BMP value can be expected after 26 days of wheat straw anaerobic digestion. S/I = 1.2 and
438 2.0 reached, respectively 43% and 39% of the BMP value (247± 8 NmL/g VS), after 15 days
439 only.

440 Most of the measured TVFA/alkalinity ratios in Experiment I (Table 3) lay within the range of
441 stable processes (0.22 to 0.36 on day 1). On day 10, S/I = 2.0, with a TVFA/alkalinity of 0.59
442 would be considered as unstable with conventional limits. However, anaerobic digestion
443 appeared to be almost unaffected. Moreover, for S/I = 3.6 and 8.5, with a TVFA/alkalinity ratio
444 close to 2 or higher, processes could be considered as strongly unstable within the usual limits. It
445 is noteworthy that S/I = 3.6 was able to recover with a TVFA/alkalinity of 1.95 whereas S/I = 8.5
446 failed with a TVFA/alkalinity of 2.46. In this experiment, a threshold of about 2 for the

TVFA/alkalinity ratio seemed to mark the difference between failure and the SSAD ability to recover.

Different studies with SSAD recorded very high TVFA/alkalinity ratios without instability or at least with the possibility for the process to recover (Table 4). Even though the VFA measurement technique can affect results (Brown and Li, 2013; Lahav and Morgan, 2004; Liew et al., 2011), the SSAD process would be the main factor that could explain a higher TVFA/alkalinity ratio. SSAD involves a very heterogeneous medium, especially regarding pH (Li et al., 2011; Martin, 2001; Staley et al., 2011), which is probably why higher S/I ratios can be observed. Acid-tolerant methanogens are of primary importance for overcoming an acidification period (Staley et al., 2011). They probably played a role in the recovery of the reactor with S/I=3.6. It is likely that a pH close to 5.5 would be much better tolerated than a pH close to 5. Finally, as some areas receive less acidic leachate, the process can be regenerated (Li et al., 2011).

Table 4. Stability limits for the SSAD process.

### 3.2 Experiment II: fungal pretreatment for SSAD

In Experiment I, the reactor with S/I = 2.0 was very little affected by acidification (in contrast to the one with S/I = 3.6), while the reactor with S/I = 3.6 managed to recover from an acidification stage. Moreover, the methane productivity per reactor volume improves with higher amounts of substrate (high S/I ratio). As a consequence, an S/I ratio of 2.9 was finally selected for Experiment II (Table 2). In this experiment, a fungal pretreated straw in non-sterile conditions (FWS) was used (Fig.2C). Since raw straw was autoclaved and inoculated with fungal colonized

miscanthus pellets, this straw was also employed for the control reactor (cMWS). Thanks to this reactor, the impact of the fungal inoculum with miscanthus pellets could be distinguished from the impact of wheat straw digestibility modifications resulting from fungal pretreatment. Finally, a reactor with miscanthus pellets (without fungi) and raw straw constituted a second control (MWS) in order to determine the impact of fungal biomass presence (in cMWS) on digestibility by comparing MWS and cMWS.

### 3.2.1 A facilitated SSAD start-up phase for pretreated wheat straw

Fig. 4. Start-up phase for Experiment II. Leachate total VFA concentration (A), leachate pH (B) and daily methane production per g of VS (C) as a function of time.

During the start-up phase, all reactors had an acidic pH (<6.5) although the degree of acidification varied among reactors (Fig. 4B). The most affected batch reactor was cMWS, which had the most acidic pH (near 5.5) and the longest low-pH period (6 days). The batch reactor presenting the least difficulties was FWS, which had the highest pH and the shortest duration of acidic conditions. Low pH directly affected the daily methane production (Fig. 4C). When the pH fell below 6.5 (day 1 for cMWS, day 2 for MWS and day 4 for FWS), a stop or a decrease in daily methane production occurred (Fig. 4C). VFA accumulation peaks were observed around day 5 for all reactors (Fig. 4A). After this day, the daily methane production and pH increased thanks to the consumption of VFA.

Alkalinity was measured in cMWS on day 5 at the VFA peak, leading to a TVFA/alkalinity ratio of 1.94 (< 2, critical value in Experiment I). A rapid recovery was observed, since the critical value was not reached and because the pH always remained above 5.5 (critical pH value).

491 Finally, total VFA concentrations in the leachate were always less than 10 g/L. This could be
492 another possible critical value, as previously discussed in Experiment I.
493
494 During the first two days, the daily methane production was maximal for cMWS, followed by
495 MWS and then by FWS. This production is very likely due to rapidly hydrolysable compounds
496 such as extractives (free sugars, organic acids, *etc*. (Liew et al., 2012)) that favoured
497 acidification. Consequently, the concentration of these compounds was highest in cMWS and
498 lowest in FWS, probably owing to the longer fungal incubation time for FWS substrate. Soluble
499 sugars in particular have a high positive impact on methane production (Monlau et al., 2012).
500 Fungal pretreatments can release soluble sugars but their proportion among soluble substances
501 has a tendency to decrease during fungal decay (Rouches et al., 2016a). Hence, acidification
502 could be enhanced following a short duration of fungal pretreatment (10 days), whereas it could
503 be limited after a longer period. Long fungal pretreatment durations would allow for a higher S/I
504 to be applied and thus would increase the methane productivity in a LBR reactor (cumulated
505 production of methane per mass of reactor).
506

507 ### 3.2.2 Influence of pretreatment on the anaerobic biodegradability

508 Anaerobic biodegradability associated to the methane yield reported for pretreated VS was 10 to
509 18% higher for FWS than for the controls (MWS and cMWS, Fig. 5A, Table 5). Even though
510 higher performance was observed during an earlier study (Rouches et al., 2016b) with *P.*
511 *brumalis* on wheat straw (40% more methane yield with 21days in lab-scale sterile conditions),
512 this result remains encouraging. Indeed, few studies have reported methane production
513 improvement following fungal pretreatment of lignocellulosic substrate (Liu et al., 2016;

514 Rouches et al., 2016a). Furthermore, these studies have generally been conducted under sterile
515 conditions at laboratory scales (Liu et al., 2016, 2017; Mustafa et al., 2017, 2016). Conversely, in
516 the current work, 400 g of autoclaved straw were treated in a non-sterile pilot reactor. Using
517 unsterilized yard trimmings treated with *Cyathus stercoreus*, Zhao et al. (2014) obtained the same
518 anaerobic biodegradability as with sterilized material (this substrate is however significantly
519 different from crop residues).

520 In the present study, methane production was investigated for FWS and compared to controls
521 (with miscanthus pellets). Maximum daily methane production was slightly higher for FWS than
522 for other reactors. Moreover, the production peak occurred on day 8 for FWS whereas the peak
523 took place on day 12 for cMWS and MWS (Fig. 4C). Thus, the methane production rate was
524 enhanced for FWS; this could be due to hydrolysis enhancement following an efficient fungal
525 pretreatment, in particular lignin degradation as concluded by Mustafa et al. (2017). However,
526 caution is required in literature as the improvement can only be due to acidification in controls.
527 Consequently, it is necessary to follow pH variations for SSAD pretreatment studies. The main
528 goal of the lignocellulosic pretreatment is an improvement of hydrolysis that is generally the rate
529 limiting step for those substrates (Monlau et al., 2013) while methanogenesis is the rate limiting-
530 step when reactors acidify. Finally, the methane content of the biogas during steady production
531 stage was similar between reactors. It reached 55-60%, in agreement with values reported in the
532 literature for the SSAD of wheat straw (Cui et al., 2011).

533

534 Table 5. Methane production in leach bed reactors at different digestion times and nitrogen and carbon
535 composition of the final digestate. [a, b, c, d, e, f, g, h] values followed by a same letter are not significantly
536 different at the 90% probability level.

537 Fungal growth on wheat straw was successful, with many white areas corresponding to the
538 mycelium of *P. brumalis* BRFM 985 and no contamination that was visible to the naked-eye.
539 However, fungal growth was not homogeneous and a longer pretreatment time seemed necessary
540 for all the straw to be pretreated (Fig. 2). The presence of large untreated areas can explain why
541 TS and VS were similar for pretreated and untreated straws (Table 1). Culture conditions need to
542 be further optimized for straw BMP (Biochemical Methane Potential) to be increased, but this
543 was not in the scope of the present study (non-sterile scale-up). Indeed pretreatment conditions
544 are just as important as an efficient fungal strain for substrate digestibility to be improved (Wan
545 and Li, 2012).

546

547 **3.2.3 Performance of combined fungal pretreatment and straw SSAD**

548 Mass losses occur during fungal pretreatment and are often not taken into account when the
549 global efficiency of the process is evaluated (Brémond et al., 2018; Tišma et al., 2018). Here,
550 mass loss was accounted for by expressing the methane production per g of initial TS (before
551 pretreatment TS). At day 127 (end of experiment), the methane yield (NmL/g TS initial) was
552 similar between reactors cMWS and FWS (around 200 NmL/g TS initial), while reactor MWS
553 (219 NmL/g TS initial) displayed the highest production (Fig. 5B and Table 5).

554 The mass loss (around 20% TS for FWS in this study) observed during fungi growth on
555 miscanthus pellets and during wheat straw pretreatment led to a 10 % decrease in the final

556 methane production yield. Mass loss was thus partially counterbalanced by an increase in
557 biodegradability.

558 If a digestion duration of 60 or 75 days is considered (Table 5), the methane production from
559 FWS is 5-6% lower than for the controls. Consequently, mass loss during pretreatment was also
560 partially compensated by a slight enhancement of the kinetics. Liu et al. (2017) observed a kinetic
561 improvement of methane production during BMP tests of diverse lignocellulosic substrates
562 treated with a *C. subvermispora* strain (ATCC 90467 or ATCC 96608). Using grounded (1 or 4
563 mm) substrate pretreated in sterile conditions, an increase in biodegradability up to 120% and to
564 36% was observed for hazel and acacia branches, respectively. Conversely, their pretreatment
565 conditions led to a decrease in biodegradability for sugarcane bagasse and barley straw. When
566 considering mass loss during pretreatment, only the methane yield of hazel branches increased
567 significantly compared with the very low methane yield, around 60 mL/gVS, of untreated
568 branches (Liu et al., 2017). In the current study, the biodegradability of straw improved
569 successfully. Even under suboptimal pretreatment conditions, the results from the current study
570 remain encouraging. With a methane yield per g of initial TS, slightly less methane (10 NmL/g
571 TS initial) is obtained for FWS than for the controls after 60 days. Nevertheless, during the first
572 twenty days, the methane production per g of initial TS is better for FWS owing to a better start-
573 up phase (Fig. 5B). Consequently, this type of pretreatment might be useful for reactors with
574 short reaction durations (<20 d) which corresponds more to continuous processes with low
575 hydraulic retention time than discontinuous ones that usually last 30 to 60 days.

576

577 Fig. 5. Main parameters of anaerobic digestion during Experiment II. (A), methane production
578 per g of substrate VS and (B), methane production relative to TS before fungal inoculation.

579

580 In addition to a better start-up phase and to improved biodegradability, another advantage of
581 fungal pretreatment for lignocellulosic biomass is the decrease of the C/N ratio due to
582 consumption of carbon and to conservation of nitrogen during pre-treatment. This reaction,
583 combined with N input by fungal inoculum, leads to an increase in the TKN content for
584 colonized substrates (Table 1), as also observed by Bisaria et al. (1983) and Zeng et al. (2011).
585 The optimal C/N ratio for anaerobic digestion lies between 20 and 35 and depends on the
586 feedstock. Higher ratios can entail nitrogen limitations, increased VFA accumulation and
587 decrease in process stability and biogas yields (Li et al., 2011; Sialve et al., 2009). Wheat straw
588 generally requires co-substrate for its C/N ratio to decrease, as it can reach values as high as 161
589 (McKendry, 2002). If nitrogen-rich co-substrates are not available on site, N-supplementation can
590 represent additional costs. However, these could be reduced if fungal pretreatment is carried out.
591 At the end of the SSAD, solid digestates have a similar NTK content, but $NH_4^+$ concentrations in
592 the leachate (Table 5) reflect the N-content of the initial substrate: FWS > cMWS > MWS (Table
593 2). Consequently, leachate produced from SSAD of fungal pretreated straw have a potentially
594 higher fertilizing value, since available nitrogen is a primary requirement for plant growth.
595 However, considering potential inhibition of anaerobic digestion by ammonia, this increase in
596 ammonium concentration may be a drawback in the case of codigestion of fungus-pretreated
597 straw with high nitrogen content substrates. Similarly, fungal pretreatment may not be applied to
598 nitrogen-rich feedstocks. Finally, a slight decrease in the final total carbon for FWS compared to

599 the controls was observed (Table 5); this can be related to the pretreatment itself and/or to the
600 more efficient anaerobic digestion.

601 ## 4 Conclusion

602 The present study points out that S/I ratios of about 2-3 are required for the SSAD of wheat straw
603 in batch leach bed reactors. It has been demonstrated that SSAD processes can recover from
604 acidification with a TVFA/alkalinity lower than 2 gHAc_eq/g$CaCO_3$, with VFA concentrations
605 lower than 10 g/L, and with a pH close to 5.5. To determine whether such limits can be
606 generalised to batch SSAD in leach bed reactors in order to better control them, studies on other
607 substrates and anaerobic inocula would be worthwhile.
608 Fungal pretreatments do not always improve methane production. In this study, a non-totally-
609 sterile pilot-scale white-rot fungi pretreatment for anaerobic digestion was applied, leading to a
610 slight improvement in wheat straw biodegradability (from 215 to 254 NmL/g $VS_{pretreated}$).
611 However, although rarely addressed in literature, pretreatment efficiency assessment should
612 include the risk of acidification during start-up which represents critical step for batch SSAD. An
613 easier SSAD start-up phase was also achieved with fungal treated straw, while the duration of the
614 fungal culture was assumed to probably influence the tendency for digesters to acidify. In the
615 current study, substrate mass losses during pretreatment led to minor methane production losses
616 (161 against 171 NmL/g $TS_{initial}$ after a 60-day anaerobic digestion), possibly because the fungal
617 culture conditions had not been sufficiently optimised. Finally, increased nitrogen availability
618 might also represent an advantage for fungal pretreated straws.
619 Fungal pretreatment could be profitable for biogas plants, especially if its cost is controlled
620 (possibility to produce fungal inoculum on site). Research efforts should continue to propose

optimized fungal pretreatment at pilot-scales. Criteria should be: low cost, possibility to treat non sterile substrate, low mass losses (especially for carbohydrates) and high delignification yields.

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787

**Figure captions**

Fig.1. Schematic diagram of laboratory-scale SSAD digesters (6 L). The inoculum/substrate mix is separated from the leachate tank with a sieve and the leachate is discontinuously recycled.

Fig. 2. Schematic representation of the fungal pretreatment. A. Pretreatment inputs. B. Aerobic pilot-reactor design for wheat straw pretreatment with Polyporus brumalis, BRFM 985. Moist air travels through the thermostatted pretreatment reactor equipped with two trays containing straw and fungal inoculum. Diam: diameter; MC: Moisture Content. C. Picture of pretreated wheat straw used in Experiment II. White areas correspond to the mycelium of P. brumalis BRFM 985.

Fig. 3. Main parameters of anaerobic digestion during Experiment I. Methane production (A), leachate pH (B), leachate total VFA concentration (C) and daily methane production (D) in function of time for several S/I ratios.

Fig. 4. Start-up phase for Experiment II. Leachate total VFA concentration (A), leachate pH (B) and daily methane production per g of VS (C) as a function of time.

Fig. 5. Main parameters of anaerobic digestion during Experiment II. Methane production per g of substrate VS (A), and methane production relative to TS before fungal inoculation (B).

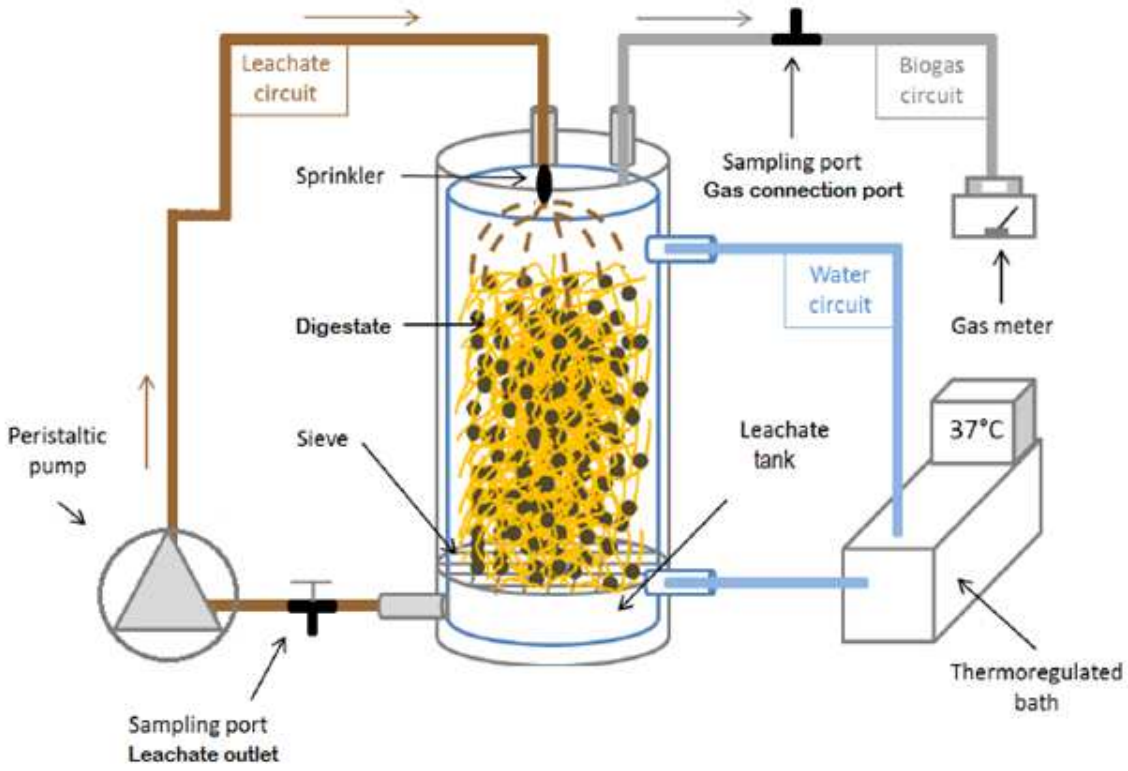
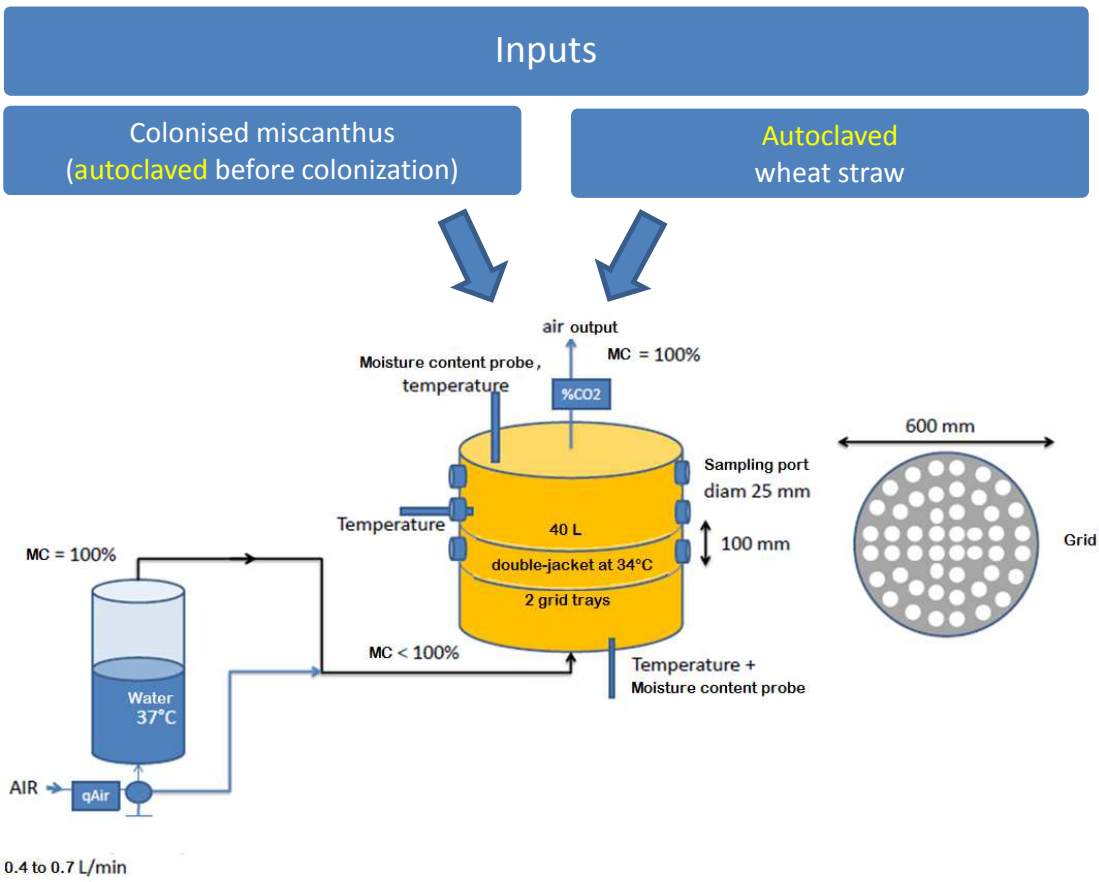



Fig.1. Schematic diagram of laboratory-scale SSAD digesters (6 L). The inoculum/substrate mix is separated from the leachate tank with a sieve and the leachate is discontinuously recycled.

A




C


Fig. 2. Schematic representation of the fungal pretreatment. A. Pretreatment inputs. B. Aerobic pilot-reactor design for wheat straw pretreatment with Polyporus brumalis, BRFM 985. Moist air travels through the thermostatted pretreatment reactor equipped with two trays containing straw and fungal inoculum. Diam: diameter; MC: Moisture Content. C. Picture of pretreated wheat straw used in Experiment II. White areas correspond to the mycelium of P. *brumalis* BRFM 985.

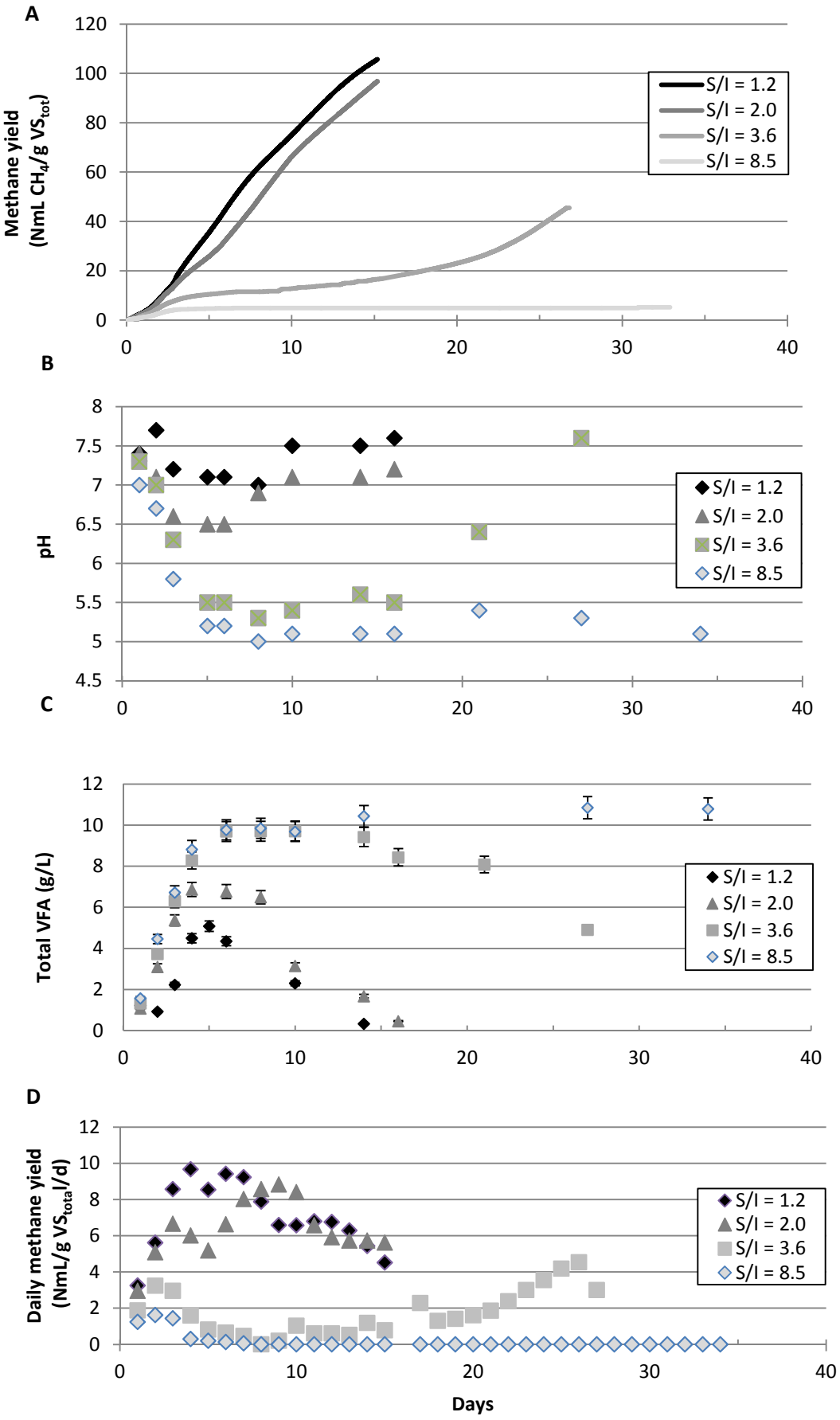
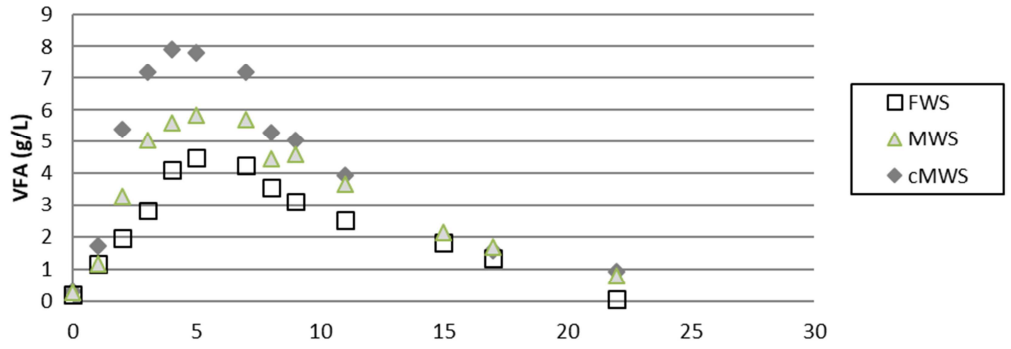
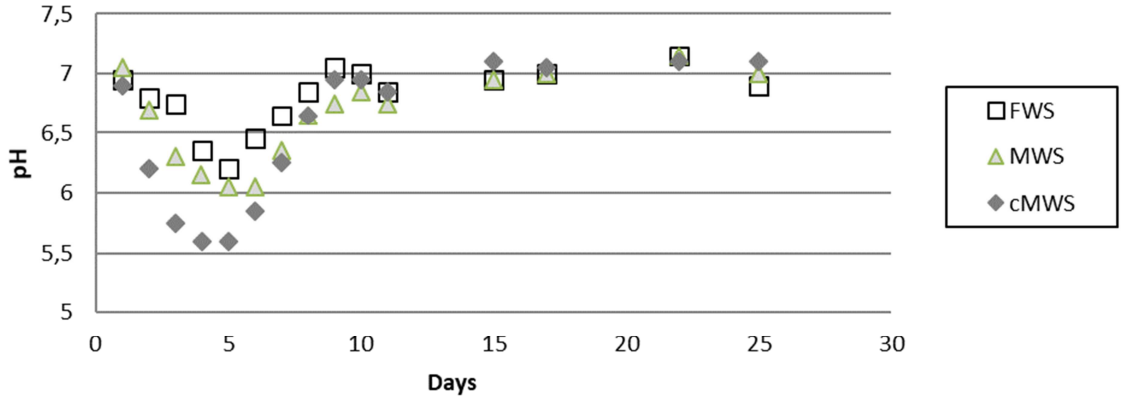



Fig. 3. Main parameters of anaerobic digestion during Experiment I. Methane production (A), leachate pH (B), leachate total VFA concentration (C) and daily methane production (D) in function of time for several S/I ratios.

A




B




C

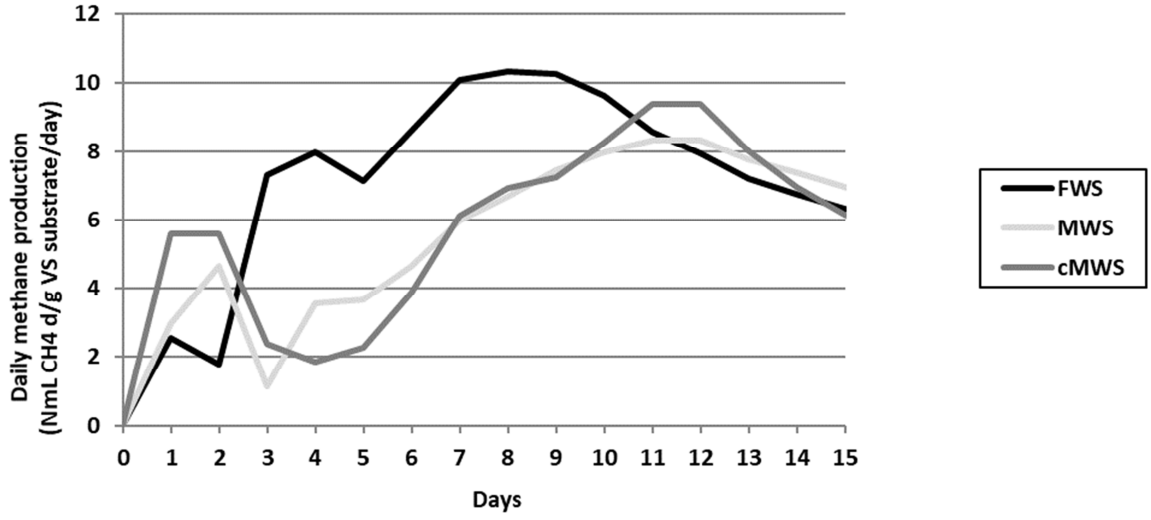



Fig. 4. Start-up phase for Experiment II. Leachate total VFA concentration (A), leachate pH (B) and daily methane production per g of VS (C) in function of time.

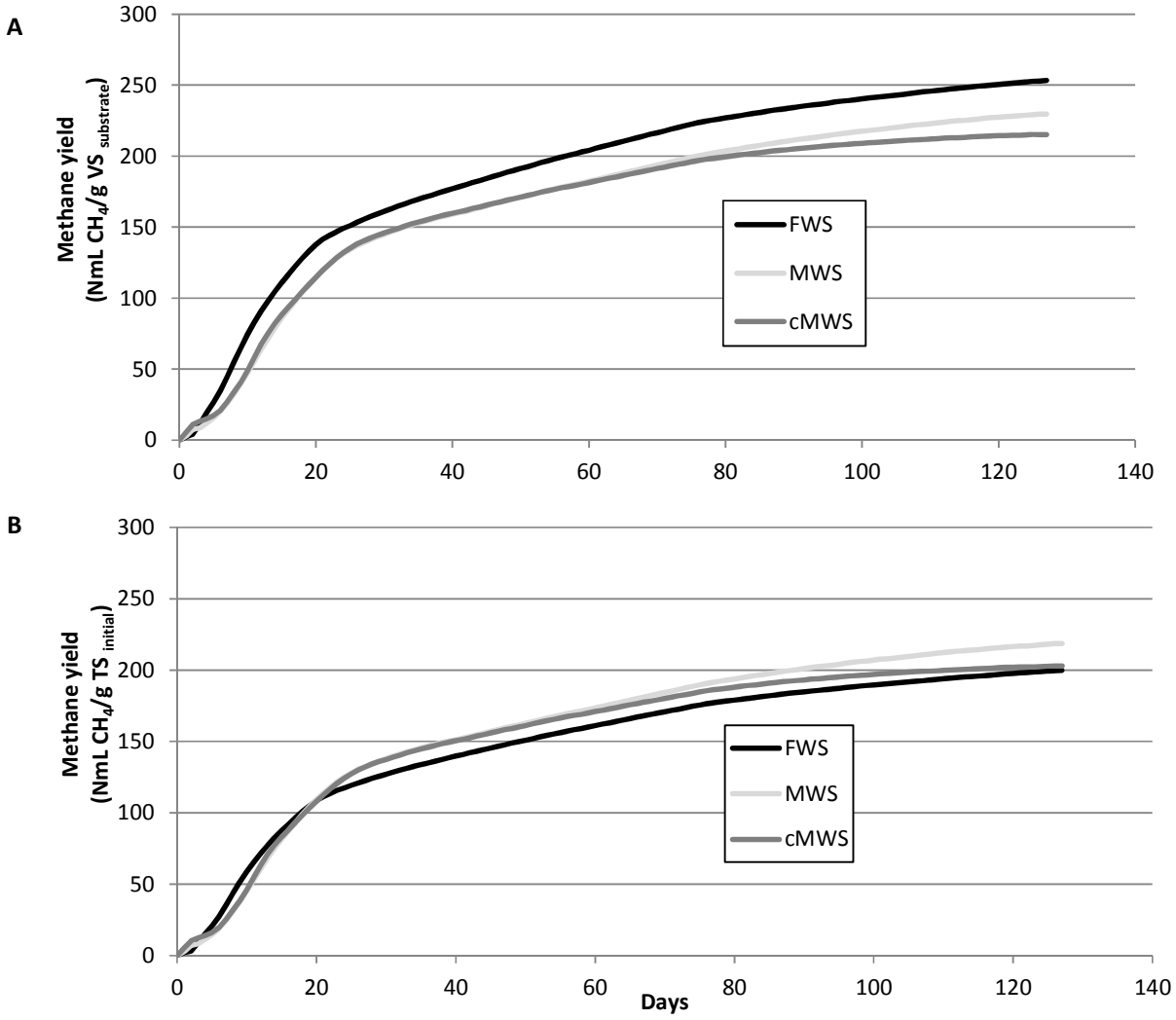



Fig. 5. Main parameters of anaerobic digestion during Experiment II. Methane production per g of substrate VS (A), and methane production relative to TS before fungal inoculation (B).

Table 1. Characteristics of substrates and inocula used in Experiments I and II. TS and VS content and total nitrogen concentrations.

| | Experiment I | | Experiment II | | |
|---|---|---|---|---|---|
| | **TS** (%wet w) | **VS** (%TS) | **TS** (%wet w) | **VS** (%TS) | **TKN** (%N) |
| Solid inoculum | 13.7±0.1 | 76.3±0.3 | 11.7±0.4 | 73.6±0.6 | |
| Liquid inoculum | 2.1±0.1 | 51.5±0.7 | 1.4±0.1 | 43.2±0.7 | |
| Wheat straw | 88.4±0.1 | 95.2±0.5 | | | |
| Autoclaved and freeze –dried wheat straw | | | 97.3±0.1 | 94.2±0.1 | 0.50±0.03 |
| Fungal pretreated wheat straw (freeze-dried) | | | 96.5±0.3 | 94.6±0.5 | 0.76±0.01 |
| Miscanthus pellets (autoclaved and freeze-dried) | | | 96.5±0.1 | 97.2±0.1 | 0.16±0.02 |
| Colonized miscanthus pellets | | | 36.7±0.6 | 96.5±0.1 | 0.13±0.01 |

Table 2. Experimental set-up for Experiments I and II.

| | | Experiment I | | | | Experiment II | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Inoculum control | Miscanthus + Straw (**MWS**) | Colonized Miscanthus + Straw (**cMWS**) | Fungal pretreated straw (**FWS**) |
| Solid inoculum | (g wet w) | 1015 | 750 | 475 | 205 | 1695 | 630 | 630 | 630 |
| $I_S$ | (g VS) | 106 | 79 | 50 | 21 | 146 | 54 | 54 | 54 |
| Wheat straw | (g wet w) | 155 | 200 | 240 | 280 | 0 | 155 | 155 | 195 |
| | (g VS) | 130 | 168 | 202 | 235 | | 141 | 141 | 178 |
| Miscanthus | (g wet w) | | | | | 0 | 39.2 | 83.5 | 0 |
| | (g VS) | | | | | | 36.7 | 29.6 | |
| %TS of water saturated substrate | | 15.5 | 16.3 | 16.8 | 17.3 | 17.7 | 18.4 | 18.3 | 17.9 |
| Total added liquid including liquid inoculum (mL) | | 2180 | 2350 | 2535 | 2720 | 1480 | 2360 | 2360 | 2360 |
| Liquid inoculum (mL) | | 600 | 600 | 600 | 600 | 1200 | 1200 | 1200 | 1200 |
| Liquid Inoculum $I_L$ (g VS) | | 6.5 | 6.5 | 6.5 | 6.5 | 7.3 | 7.3 | 7.3 | 7.3 |
| Inoculum I ($I_{S+}I_L$) (g VS) | | 112.5 | 85.5 | 56.5 | 27.5 | 153.3 | 61.3 | 61.3 | 61.3 |
| Proportion of solid inoculum $I_S$ /($I_{S+}I_L$) (%VS basis) | | 94 | 92 | 88 | 76 | | 88 | 88 | 88 |
| **S/I (VS basis)** | | **1.2** | **2.0** | **3.6** | **8.5** | | **2.9** | **2.8** | **2.9** |

Table 3. Alkalinity, TVFA/alkalinity and pH on days 1 and 10 of the Experiment I. Parameters at day 10 for S/I=3.6 led to a recovery of methanogenic activity whereas for S/I= 8.5 the inhibition persisted.

| | Day 1 | | | Day 10 | | |
|---|---|---|---|---|---|---|
| **S/I** *VS basis* | **Alkalinity (TA)** *g $CaCO_3$/L* | **TVFA/alkalinity** *gHAc_eq/g$CaCO_3$* | **pH** | **Alkalinity (TA)** *g $CaCO_3$/L* | **TVFA/ alkalinity** *gHAc_eq/g$CaCO_3$* | **pH** |
| 8.5 | 4.24 | 0.36 | 7 | 3.28 | 2.46 | 5.1 |
| 3.6 | 3.87 | 0.33 | 7.3 | 4.12 | 1.95 | 5.4 |
| 2.0 | 4.67 | 0.22 | 7.3 | 4.57 | 0.59 | 7.1 |
| 1.2 | 4.40 | 0.27 | 7.3 | 4.83 | 0.40 | 7.3 |

Table 4. Stability limits for SSAD process.

| **TVFA/alkalinity** | **Anaerobic digestion process** | **Substrate** (composition in %TS) | **Additionnal information** | **Reference** |
|---|---|---|---|---|
| Final ratio at **1.6** or above for good performance reactors | Mesophilic **batch** at **20%TS** at S/I (VS basis) ratio of 4.1 or 6.2 for 30 d, no shaking | Fallen leaves (11.1% CEL, 11.5% Hemi, 22.7% LIG) | 0, 2, 3.5 or 5% NaOH addition at the inoculation step | (Liew et al., 2011) |
| Ratio followed during the whole process:<br>• Stable SSAD when **< 0.9**<br>• Acidification and recovery when **> 1.25 and < 5.4**<br>• Satisfactory methane production even with a peak between **3 and 4** | Mesophilic **batch** at **20%TS** at S/I of 2.2 (VS basis) for 48 d, slow shaking | Single or co-digestion of distiller's grain (22.9% Hemi, 19.9% Cel, 13.8% LIG) and food waste (12.3% Hemi, 4.4% Cel, 2.8% LIG) with several proportions tested | Initial alkalinity of 10 g/L $CaCO_3$ thanks to $NaHCO_3$ and $KHCO_3$ addition | (Wang et al., 2012) |
| Initial ratio between **0.9 and 1.2** and**:**<br>• Final ratio **< 3** for reactors producing satisfactory methane<br>• Final ratio **> 3** for acidified reactor with no or very low methane production | Mesophilic **batch** at **20%TS** for 30 d at S/I (VS basis) of 1, 2 or 3 | Mix of yard (24.3% Cel, 23% LIG, 9.7% Hemi) and food wastes (0, 10 or 20% VS) with several proportions tested | | (Brown and Li, 2013) |
| Peak around **3**, average value of **2**, stable process (methane production and pH) | Thermophilic **continuous** process (stirred tank reactor, leachate recycle) at **20%TS** and 30% (w/w) inoculum for 60 d | Food Wastes from restaurant | | (Forster-Carneiro et al., 2008) |
| A decrease of organic loading rate allowed the recovery of stable process with satisfactory methane production after a peak between **0.8 and 1.1** | Mesophilic semi-**continuous** process (stirred tank reactor) at **15% or 20% TS** | Dewatered sewage sludge | | (Duan et al., 2012) |
| • Possible recovery with a peak value of **1.95**<br>• Failure with a peak at **2.49** | Mesophilic **batch** with leachate recycle at **17-18%TS** S/I (VS basis) of 3.6, 8.5 and 2.9 | Wheat straw inoculated with manure digestate | | Current study |
| • Peak value at **1.94** with a satisfactory methane production | | Fungal colonized miscanthus and wheat straw | | |

Table 5. Methane production in leach bed reactors at different digestion times and nitrogen and carbon composition of the final digestate. [a, b, c, d, e, f, g, h] values followed by a same letter are not significantly different at the 90% probability level.

| | | Inoculum control | Miscanthus + Straw (**MWS**) | Colonized Miscanthus + Straw (**cMWS**) | Fungal pretreated straw (**FWS**) |
|---|---|---|---|---|---|
| **Substrate biodegradability** | | | | | |
| $CH_4$ production (NmL/g VS substrate) | Day 60 | | 181 | 181 | 204 |
| | Day 75 | | 196 | 196 | 222 |
| | Day 127 | | 230 | 215 | 254 |
| **Process performance** | | | | | |
| ($NmLCH_4$/g TS initial) | Day 60 | | 173 | 171 | 161 |
| | Day 75 | | 187 | 185 | 177 |
| | Day 127 | | 219 | 203 | 200 |
| **Final** TKN for solid digestate | (%TS) | 2.37±0.04[d] | 2.40±0.06[d] | 2.44±0.07[d] | 2.36±0.09[d] |
| **Final** $NH_4^+$ in leachate | (mg/L) | 9.53±0.02[e] | 5.52±0.02[f] | 9.20±0.01[g] | 18.26±0.04[h] |
| **Final** TOC for solid digestate | (%TS) | 37.61±0.06[a] | 44.9±0.5[b] | 44.9±0.4[b] | 43.0±0.9[c] |