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Title: Semi-continuous Mono-digestion of OFMSW and Co-digestion of OFMSW with Beech Sawdust: Assessment of the Maximum Operational Total Solid Content

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Abstract: In this study, mono-digestion of the organic fraction of municipal solid waste (OFMSW) and co-digestion of OFMSW with beech sawdust, simulating green waste, were used to investigate the maximum operational total solid (TS) content in semi-continuous high-solids anaerobic digestion (HS-AD). To alleviate substrate overloading in HS-AD, the effluent mass was relatively reduced compared to the influent mass, extending the mass retention time. To this aim, the reactor mass was daily evaluated, permitting to assess the reactor content removal by biogas production. During mono-digestion of OFMSW, the NH₃ inhibition and the rapid TS removal prevented to maintain HS-AD conditions (i.e. TS ≥ 10 %), without exacerbating the risk of reactor acidification. In contrast, the inclusion of sawdust in OFMSW permitted to operate HS-AD up to 30 % TS, before acidification occurred. Therefore, including a lignocellulosic substrate in OFMSW can prevent acidification and stabilize HS-AD at very high TS contents (i.e. 20-30 %).

Semi-continuous Mono-digestion of OFMSW and Co-digestion of OFMSW with Beech Sawdust: Assessment of the Maximum Operational Total Solid Content

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2nd May 2018

Prof. R. Dewil,

Co-Editor-in-Chief

Journal of Environmental Management

Cover letter - manuscript submission

Dear Prof. R. Dewil,

Please find attached the manuscript with the title “*Semi-continuous Mono-digestion of OFMSW and Co-digestion of OFMSW with Beech Sawdust: Assessment of the Maximum Operational Total Solid Content*” by Vicente Pastor Poquet, Stefano Papirio, Eric Trably, Jukka Rintala, Renaud Escudié and Giovanni Esposito.

High-solids anaerobic digestion (HS-AD) is a well-established treatment technology for the organic fraction of municipal solid waste (OFMSW), being operated at a total solid (TS) content ≥ 10 %. Thus, HS-AD enhances the overall economy of the process, while contributes to the abatement of the uncontrolled greenhouse gases emissions associated to the landfilling of OFMSW.

The semi-continuous HS-AD reactor setup is widely used for OFMSW treatment. However, the design and operation of HS-AD reactors strongly depend on the OFMSW composition and/or the inclusion of lignocellulosic substrates to OFMSW, which might be associated to regional/seasonal variances and/or the local strategies for waste management (i.e. recycling). Moreover, since the early HS-AD studies for OFMSW treatment (i.e. back to the 80’s), the increasing awareness about sustainable development and global warming have triggered the implementation of new policies and/or waste management strategies, influencing the OFMSW composition. For example, a progressive reduction in the inert content (i.e. plastics, metals) of OFMSW has been observed in Europe during the last 30 years associated to the European Waste Framework Directive.

Therefore, understanding the effect of the OFMSW composition to determine the TS content in semi-continuous HS-AD reactors is crucial to take advantage of the benefits of this biotechnology. In this scheme, the laboratory-scale experiments permitted to highlight invaluable aspects of semi-continuous HS-AD as, for example, the need to reduce the effluent in comparison to the influent to maintain the reactor content constant, extending the biomass retention time and avoiding the reactor failure.

The present study relates the OFMSW composition with the goal of maintaining a high operating TS content in laboratory-scale semi-continuous HS-AD reactors. The results suggest that the maximum TS to be used in HS-AD depends on the OFMSW biodegradability and presence of inhibitory compounds (i.e. NH_3), being the addition of lignocellulosic substrates an adequate

strategy to overcome acidification and stabilize HS-AD of OFMSW at very high TS contents (i.e. 20-30 %).

The authors consider that the present study address the *Journal of Environmental Management* requirements, regarding the implementation of environmental technologies for organic waste treatment and resource recovery. Particularly, the study links together the OFMSW composition, the process economy and operation, and the NH₃ inhibition, being this information particularly important for scientists and engineers, as well as for the development of mathematical models for HS-AD of OFMSW.

All the authors mutually agreed to submit this manuscript to *Journal of Environmental Management*. We confirm that the manuscript is an original work, that it has not been previously submitted to the *Journal of Environmental Management*, and that it is not under consideration for publication anywhere else.

We kindly thank you for your time and consideration. In case you need to contact me, please feel free to use the below address, phone or e-mail address.

Sincerely yours,

Vicente Pastor Poquet

On behalf of all the co-authors

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RESPONSE TO THE EDITOR'S AND REVIEWERS' COMMENTS

Title: Semi-continuous Mono-digestion of OFMSW and Co-digestion of OFMSW with Beech Sawdust: Assessment of the Maximum Operational Total Solid Content

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Date: 7th September 2018

Dear reviewers and manuscript editor,

We would like to express our sincere appreciation for all the time and effort that you devoted to the examination of this manuscript. All the comments have been carefully considered and amended accordingly. Please, find below the due answers and proposed modifications for each of your previous comments. We believe that after these modifications, the manuscript has enhanced its quality and readability considerably. Please, do not hesitate to contact me/us any time you consider it necessary.

Sincerely Yours,

Vicente Pastor Poquet

On behalf of all the co-authors

Editor Recommendation: “Due to space limitations in the printed journal, we are requesting that all authors reduce the length of their papers by at least 10% if possible. If your paper includes large tables or datasets, it is preferred that these be published as supplementary material in Science Direct rather than in print”.

Following with the recommendation for reducing the length of the manuscript, several References from the original manuscript have been removed, while Table 1 has been moved to Supplementary Information (as Table A1). Importantly, removing some references did not affect the overall content of the manuscript. Those references were

also modified to address the first comment of Reviewer #2. Please see these comments below. Meanwhile, the corrections proposed by the reviewers required extending slightly the manuscript. Thus, all these corrections were kept as short as possible, while some minor modification thorough the text permitted also to reduce slightly the length of the manuscript. The authors consider that reducing further the manuscript might result in a loose of important information. In either case, we could address any further suggestion in this regard, whether the editor and/or reviewers consider it necessary.

Lines 211-212 previously stating: “A summary of the weekly operational variables is presented in Table 1”

were substituted by

“A summary of the weekly operational variables is presented as Supplementary Information”.

Meanwhile, Table 2 and Table 3 were renamed as Table 1 and Table 2 throughout the manuscript.

With all the above, the original version of this manuscript contained 7777 words (including 45 references) + 3 tables + 5 figures.

In this version, after addressing all the due corrections, the manuscript contains 7991 words (but only 36 references) + 2 tables + 5 figures.

Reviewer #1: This is an interesting study. Like many in this area, it is quite empirical and observational and does not address underlying mechanism. I do not think this is a reason for rejecting the work since the results are, nevertheless, interesting.

I suggest that the authors incorporate some statistical treatment of the data - for example:

a. Include some details in the Methods about how the data were treated statistically

To address this comment, a new subsection within the methodology was added in Lines 254-258:

“2.5 Statistical Analyses

The Dixon’s test for BMP outliers was applied as recommended by Holliger et al. (2016). The unpaired t-test of Microsoft Excel 2016 (Microsoft, USA) was applied to determine the statistical significance of experimental data, using the two-tail p-value at 95 % confidence.”.

b. In Tables 2 and 3 how were the errors estimated? Why do only some values have errors estimated?

In this study, results in Tables 2 and 3 were averaged and the standard deviation calculated with Excel[®]. On the other hand, the BMP values were calculated as recommended by Holliger et al. (2016).

In this manuscript version, the BMP values in Table 2 have been updated, since BMP outliers have been removed as also recommended by Holliger et al. (2016).

BMP values have been also modified in Lines 269-270 and Line 434.

Meanwhile, in Table 3, since only TS and VS of the co-digestion inoculum were obtained in duplicate, the standard deviation of these two analyses has been removed to homogenize the results format.

c. Where appropriate, use statistical tests to compare values

Please, see comment (a) above. In this manuscript version, the unpaired t-test has been carried for all the previously-used comparisons between semi-continuous reactors.

Particularly, p-values have been added in Lines 302-303, 305-306, 322-325, 358-359, 360-361, 366-367, 374-375, 440-442 and 504-505.

Reviewer #2: The aim of the current manuscript by Pastor-Poquet et al was to investigate the high solids-AD performance using the OFMSW and the addition of a green lignocellulosic waste (sawdust) as a strategy to achieve a more stable and efficient biogas production. The work presents results including TS, nitrogen, % CH₄ and VFA to support the performance of the AD processes in two groups of reactor, including mono and co-digestion. The results provided are interest to the wider readership of the journal, since its interesting approach. Nevertheless, it is my opinion that addressing a few points will improve the overall quality of the manuscript. They are as follows:

General recommendations:

- There are many references from 1990, 1995 and 200 for example. Actually, references from the past 15 years represents 40% of the overall references used. I recommend updating them, since many interesting contributions were published after this time.

The summary of references within the original manuscript version was:

No. References = 45

Year ≤ 2000 → 15 Ref. (33 %);

Year ≤ 2005 → 24 Ref. (53 %);

Year ≤ 2008 → 30 Ref. (66 %);

In this version, the references summary is as follows:

No. References = 36

Year \leq 2000 \rightarrow 5 Ref. (14 %);

Year \leq 2005 \rightarrow 12 Ref. (33 %);

Year \leq 2008 \rightarrow 18 Ref. (50 %);

As it can be observed, several references from year \leq 2000 have been removed and/or updated as recommended. Importantly, all the added references condense the same information of the previously-used references. On the other hand, some references were also added to support some of the information needed to address all the rest of comments throughout this revision document.

- There are many interesting data regarding reactor performance, so I strongly believe that a table including the main results (averages from a week with the same OLR for example) would helping the readers understanding the specific discussions.

All coauthors agree that including more experimental data could enhance the quality of the manuscript. However, it would extend also the length of the manuscript. Therefore, to address this particular comment, 4 more tables were added as Supplementary Information. In Tables A2 and A3 the weekly-averaged results of 7-d OLR and 7-d MRT are presented for mono-digestion and co-digestion reactors, respectively. On the other hand, Tables A4 and A5 include the weekly averaged dynamic variables (i.e. TS, VS, pH, TAN, NH₃, total VFA and CH₄ content) for mono-digestion and co-digestion reactors, respectively. These tables could be further modified and/or included within the due manuscript, whether the manuscript editor and/or reviewers consider it necessary.

Noteworthy, in Lines 285-286, the following sentence was also added: “The weekly-averaged results were also included as Supplementary Information”.

Since Tables A2-A5 contain all the experimental results until Week No. 16, Table A1 (previously Table 1) was extended accordingly to ease comparison of results.

- Please add a comma (,) in the thousand units in the text and figures.

We have modified the VFA units from mg/kg to g/kg throughout the text.

In Lines 277 to 279, “2300 and 3000 mg/kg” was substituted by “2.30 and 3.00 g/kg”.

In Line 281, “20 mg/kg” was substituted by “0.02 g/kg”.

In Line 473, “3000 to 9000 mg/kg” was substituted by “3.00 to 9.00 g/kg”.

In Line 475, “700 mg/kg” was substituted by “0.70 g/kg”.

In Line 476, “150 mg/kg” was substituted by “0.15 g/kg”.

In Lines 476-477, “5000, 4000 and 1100 mg/kg” was substituted by “5.00, 4.00 and 1.10 g/kg”.

In Line 478, “3000 to 5000 mg/kg” was substituted by “3.00, to 5.00 g/kg”.

In Line 482, “4000 mg/kg” was substituted by “4.00 g/kg”.

In Line 483, “1200 (day 7) to 5800 mg/kg” was substituted by “1.20 (day 7) to 5.80 g/kg”.

In Line 490, “2700 to 5800 mg/kg” was substituted by “2.70 to 5.80 g/kg”.

In Line 494, “8300 mg/kg” was substituted by “8.30 g/kg”.

In Lines 494-495, “360 mg/kg” was substituted by 0.36 g/kg”.

In Line 497, “8200 mg/kg” was substituted by “8.20 g/kg”.

In Line 498, “350 mg/kg” was substituted by “0.35 g/kg”.

In Line 499, “7200 mg/kg” was substituted by “7.20 g/kg”.

In Line 501, “1000 to 3700 mg/kg” was substituted by “1.00 to 3.70 g/kg”.

In Lines 501-502, “500, 140 and 5 mg/kg” were substituted by “0.50, 0.14 and 0.00 g/kg”

In Lines 502-503, “3000-3500, 2900-3200 and 2500-2600 mg/kg” was substituted by “3.00-3.50, 2.90-3.20 and 2.50-2.60 g/kg”.

Noteworthy, in Figures 3 and 5, the VFA units have been modified from mg/kg to g/kg, and the figures slightly enlarged.

Specific recommendations:

Key-words: rep;ave OFMSW since it already appears on the title.

In this version, Keyword “OFMSW” has been substituted by “Acidification”.

P6 L3: The substrate were able to homogenization by itself? Was there any difficulties by doing so or extra water was added? It is important to register it.

To address this comment, Line 114 previously stating

“fully homogenized and stored in 5L buckets”

was substituted by

“fully homogenized manually and stored in 5L buckets”.

On the other hand, the following sentence was also included in Lines 115-116: “During mincing and homogenization, no extra water was added to the raw substrate”.

P6 L16: Is it possible to provide the OFMSW and inoculum collection point (city, names...)?

To address this comment, Lines 109-111 previously stating

“OFMSW consisted of a mixture of household waste, restaurant waste, spent coffee, and garden waste”

were substituted by

“OFMSW consisted of a mixture of household waste collected in Cassino (Italy), restaurant waste, spent coffee, and garden waste collected at the university facilities”

Similarly, Lines 123-124 previously stating

“sludge collected from a mesophilic (35°C) digester treating buffalo manure and mozzarella whey”

were substituted by

“sludge collected from a mesophilic (35°C) digester treating buffalo manure and mozzarella whey (Capaccio, Italy)”.

P6 L33: Is there any explanation for this 1 month period without feeding the 'source reactor? It seems that the 7 and 15 days from feeding restart was used for degassing period (which is more than necessary), so it is recommended to explain this 'extra' 30 days.

To address this comment, Lines 128-131 previously stating

“Prior to start the mono-digestion experiments, the source reactor was kept unfed for 1 month and, subsequently, the feeding with diluted OFMSW was resumed to recover the methanogenesis activity”

were substituted by

“Prior to start the mono-digestion experiments, the source reactor was kept unfed for 1 month and, to consume/reduce the organic content, while continuing with the inoculum adaptation to the new substrate. Subsequently, the feeding with diluted OFMSW was resumed to recover methanogenesis”

P7 L35: It is better to change 'or' to 'and' since the reactors were all operated at the same time.

Dear reviewer, we apologize but we consider that the word ‘or’ condenses better the fact that mono-digestion and co-digestion reactors were not operated at the same time. Thus, in Lines 138-140, it was previously mentioned that “Once the mono-digestion experiments ended, the source reactor was kept unfed for 1 month to serve as inoculum for the co-digestion experiments”.

P7 L40: I recommend: "The semi-continuous reactors and reactor influents/effluents were weighted on a ± 0.01 precision scale."

Lines 156-159 previously stating

“The semi-continuous reactors were weighted on a ± 0.01 kg precision scale, before/after the discharge/loading operations, while the reactor influents/effluents were weighted on a ± 0.01 g precision scale”

were substituted by

“The semi-continuous reactors (i.e. kg) and the reactor influents/effluents (i.e. g) were weighed on a ± 0.01 precision scale”.

P7 L45: add OLR unit

Lines 159-163 previously stating

“The OLR was evaluated as the daily VS substrate addition divided by the reactor mass content, while the mass retention time (MRT) was evaluated as the quotient between the reactor mass and reactor effluent mass”

were substituted by

“The OLR was evaluated as the daily substrate addition in terms of volatile solids (VS) divided by the reactor mass content (i.e. g VS/kg·d), while the MRT was evaluated as the quotient between the reactor mass and the daily effluent mass (i.e. days)”.

P7 L50: add MRT unit

Please, see previous comment.

P8 L33-45: This paragraph is quite confusing. I recommend clarifying the ideas here or adding an extra explanation closer to the results.

To address this comment, Lines 186-190 previously stating

“The methanogenic activity was roughly associated to the relative increase of the pH and inorganic carbon alkalinity (ALK_P), the reduction of the reactor mass content and the biogas production compared to previous operational values”

were substituted by

“In each reactor, the methanogenic activity was roughly associated to the relative increase of the pH and inorganic carbon alkalinity (ALK_p), the reduction of the reactor mass content and the biogas production compared to previous operational values, as also mentioned in section 3.2”.

Meanwhile, Lines 292-293, previously stating

“As pH recovered in reactor A (i.e. pH = 7.6, day 29), feeding was also resumed”

were substituted by

“As pH recovered in reactor A (i.e. from 6.4 on day 21, to 7.6 on day 29) due to methanogenesis activity, feeding was resumed.”.

Similarly, in Lines 293-295, the following sentence was also added: “During the same period, ALK_p in reactor A increased alongside pH from 1.1 to 2.7 g $CaCO_3/kg$ (data not shown), as an indicator of ongoing methanogenesis”.

Finally, in Lines 320-322, the following sentence was also added: “As an example, the occurrence of methanogenesis led to a 60 g removal of the reactor mass content in both mono-digestion reactors from day 37 to 41 (data not shown).”.

Table 1: Why the mass added in the reactors A and B for mono and A, B and C for co-digestion are different throughout the same week if there were to be operated at the same conditions?

In this study, parallel reactors were conceived to be operated under slightly different conditions, particularly regarding the use of a relatively higher OLR, to observe potential differences in the reactor performance. For example, in Lines 302-304, it was mentioned that “The OLR in (mono-digestion) reactor B was averagely about 1.5 g VS/kg·d higher than that used in reactor A during the whole experiment, explaining the relatively faster acidification observed in reactor B”.

To address this comment, Lines 183-186 previously stating

“Mono-/co-digestion reactors were fed in parallel though different OLR/MRT were used, as shown in section 3.2”

were substituted by

“To evaluate the differences in the reactor performance, mono-digestion reactors were fed in parallel using different OLR/MRT in each reactor, as shown in section 3.2. Subsequently, co-digestion reactors were also operated in parallel at three different OLR/MRT”.

P10 L8: Why extra water was used for the BMP test of OFMSW, since the mass of substrate could be reduced to keep the same ISR? Why NaHCO₃ was added? Please provide the guidelines used as a reference here.

In Lines 234-240, the following paragraph was added: “BMP tests were performed according to Angelidaki and Sanders (2004) and Holliger et al. (2016). In the BMP test for OFMSW, the distilled water and NaHCO₃ addition served to minimize the chances of inhibition (i.e. by NH₃) and acidification, respectively. In contrast, NH₃ build-up and acidification were not expected in the BMP test of sawdust, due to the low nitrogen content and the reduced biodegradability of sawdust, as thoroughly discussed in next section, permitting also to use a lower ISR”.

P10 L18: Why ISR of OFMSW and sawdust were different? The biological activity of the inoculum was evaluated using a reference sample?

Please, see previous comment.

In Lines 242-243, the following sentence was added: “Inoculum activity assays using a reference substrate were not performed”.

Table 2: How the alkalinity of the solid substrate (sawdust) was determined? Provide a simple procedure here since it is not very usual.

In this study, solid and semi-solid samples were previously diluted as recommended by the EPA (2015) guidelines. Thus, to address this comment, Lines 215-222 previously stating

“The pH, TS, volatile solids (VS), total Kjeldahl (TKN) and ammonia (TAN) nitrogen, and the total hydrogen sulfide were determined by the standard methods (APHA, 1999). The partial (ALK_P) and intermediate (ALK_I) alkalinity were determined as proposed by Lahav et al. (2002)”

were substituted by

“The pH, ALK_P and ALK_I were determined from the supernatant of solid and semi-solid samples, as proposed by Lahav et al. (2002), after diluting the sample with distilled water, homogenization and centrifugation at 6000 rpm for 15 min (EPA, 2015). The TS and VS content, total Kjeldahl (TKN) and ammonia (TAN) nitrogen, and the total H_2S were determined by the standard methods (APHA, 1999)”.

P11 L13: Minor modifications were related to the analytical methods or the results obtained? Please clarify this topic.

The small differences in the physical-chemical analysis for OFMSW (i.e. TS, TKN and BMP) were associated to the waste heterogeneity. Thus, TS and TKN were normally conducted in duplicate, while 6 replicates were used for the BMP tests. To address this comment, Lines 271-274 previously stating

“Minor modifications observed in the OFMSW characterization were attributed to the substrate heterogeneity”

were substituted by

“Despite the thorough mincing and homogenization, minor modifications were observed in the OFMSW characterization (i.e. TS, TKN or BMP), mainly attributed to the substrate heterogeneity”.

P11 L42-57 and P12 L1-13: How the authors explain the effect of no operation for 2 days a week? The lack of feeding during this period can create special conditions regarding 'reactor recovering' in terms of VFA consumption (see Fig 3a and Fig 3b) and this trend is not being showed in the figures. How the average 5days to 7 days can really avoid this? Moreover, if pH was under 6.5 and CH₄ content under 40% why data are showed in the figures after day 78, since there was no feeding? It seems these extra data do not say much.

The absence of feeding in AD normally triggers an increase in pH, TAN, %CH₄ and biogas production, while TS and acetate decrease. Thus, the influence of various days with no operation can be ‘easily’ spotted within the data trends, particularly in the rapid pH increases.

Meanwhile, 7-d-averaged OLR and MRT were thought to be more convenient operative parameters, since the cascade of bio-physical-chemical processes occurring in the digester at different time-scales normally results in a delay response in biogas production and/or VFA buildup. Moreover, a 7-d-average ‘smooths’ the digester operational parameters (i.e. OLR and MRT), easing the comparison among reactors operated under slightly different conditions. In short, a 7-d-average condenses both the operation of that particular operation day, but also the 6 days immediately preceding. Using moving-average is a common practice in environmental modelling of wastewater treatment plants and/or weather events.

On the other hand, even if after day 78 (approx.) there was no feeding, operation days from 78 to 100 were used to assess potential recovery strategies for these semi-continuous reactors, as condensed in Lines 209-211 (“Semi-continuous reactors were fed until acidification occurred. From this point, feeding was stopped and reactor

dilution and/or inorganic salt addition (i.e. NaHCO_3 and FeCl_2) were tested as recovering strategies”). The recovering strategies are also explained in [Section 3.5](#).

To address this particular comment, in [Lines 164-169](#), the following paragraph was added:

“Moving-average operational variables are well suited indicators of the immediately preceding operations (i.e. feeding, dilution, reactor content removal) to discern about the risk of VFA buildup in semi-continuous digesters. Moreover, expressing the operational conditions as a moving-average eases the comparison of digesters, when feeding days are not the same or an important mass removal occurs”.

Meanwhile, in [Lines 511-518](#), the following paragraph was also added:

“Feeding the reactors a maximum of 5 days per week influenced the reactor dynamics, since pH increased and VFA – mainly acetic acid – decreased during the periods with no feed. The pH and VFA modifications [Figure 3 and 5] were associated to the TS removal, as mentioned before, and also affected the biogas production/composition, and the TAN buildup [Figures 2 and 4]. As an example, in co-digestion reactor A, pH increased from 7.0 to 7.9 from day 37 to 41, while acetic acid decreased from 4.30 to 2.40 g/kg, triggering a biogas production of 0.6 L/kg Reactor Content and a methane content increase from 59 to 70 % [Figure 4 and 5]”.

Figure 4a: Why there are two lines for both reactors A, B and C? The second group of lines are related only to OFMSW in the mixture? Express that in the subtitle.

[Figure Caption 4a](#) previously indicating

“Organic loading rate”

was substituted by

“Organic loading rate – parentheses indicate the sole addition of OFMSW”.

P14 L16-18: Same here. Why the results after day 76 on reactors A, B and C are important since there is no feeding after this time.

Operation days from 78 to 100 were used to assess potential recovery strategies for these semi-continuous reactors, as mentioned in the second comment above.

P18 L21-45: Too general discussion of the results. I recommend eliminating this topic and discuss only data trend.

In the revised version of this manuscript, Lines 460-470 have been removed as recommended.

P20 L23: Change 'Reactor Content' to 'reactor content'

Capitalized “Reactor Content” was substituted by “reactor content” in Lines 192-193, 522, 524-525, 543 and 546-547.

Topic about recovering strategies: Why NaHCO₃ and FeCl₂ were used specifically? Had the authors consider using a trace elements solution instead of single compound? Please add in the figures the specific times when extra buffering solution was added. You can use an arrow for example.

The following sentence was added in Lines 566-568: “Adding NaHCO₃ is normally used to counteract acidification when digesters show a reduced ALK_P (Chen et al., 2008; Holliger et al., 2016)”.

The following sentence was also added in Lines 575-578: “Both Fe²⁺ and/or Fe³⁺ can be used to precipitate sulfide in AD, but Fe²⁺ was preferred in this study to avoid the

inclusion of a strong electron acceptor (i.e. Fe^{3+}) that could react with organic compounds in the anaerobic digester (i.e. $\text{Fe}^{3+} + 1/2 \text{H}_2 \rightarrow \text{Fe}^{2+} + \text{H}^+$, $\Delta G^{\circ} \ll 0$) (Fermoso et al., 2015; Rittman & McCarty, 2001).

The following paragraph was also added in Lines 599-601 “Another strategy to prevent reactor acidification and/or enhance the digester performance is trace element (i.e. Se, Ni, Co, Mo, W) addition (Fermoso et al., 2015; Mancini et al., 2018). However, this was out of the scope of this manuscript”.

In Figure 2 and 3, arrows representing the NaHCO_3 , FeCl_2 or inoculum additions were added.

In Figure Captions 3 and 4 the following sentence was also added: “Black arrows represent the addition of NaHCO_3 in reactor A, while dotted arrows represent the FeCl_2 or inoculum addition in reactor B”.

Conclusions: This topic must be improved. The comparison between reactors performance regarding the influence of sawdust addition could really be evaluated in terms of 'specific biogas production' (L biogas kg VS-1 added), since operation conditions were very different in terms of strategy.

To address this comment, Lines 659-662 previously stating “Overloading was related to the substrate biodegradability and inhibitory content, since the maximum TAN content was 4.8 and 3.6 g N/kg, while the biogas production was 166-192 and 59-71 L/kg VS Fed, for mono-digestion of OFMSW and co-digestion of OFMSW and sawdust, respectively” were removed.

Instead, Lines 649-654 previously stating

“In this study, reducing the effluent compared to the influent mass (i.e. 1-38 %) permitted to extend the biomass retention time in semi-continuous mono-digestion of OFMSW. However, the sole implementation of influent/effluent uncoupling was not

sufficient to avoid reactor overload/acidification when reaching HS-AD conditions (i.e. $TS \geq 10\%$)”

were substituted by

“In this study, reducing the effluent compared to the influent mass (i.e. 18 %) permitted to extend the MRT in semi-continuous mono-digestion of OFMSW, and obtain a specific biogas production of 229 L/kg VS added, due to the high biodegradability of OFMSW. However, the sole implementation of influent/effluent uncoupling was not sufficient to avoid reactor overload and acidification when reaching HS-AD conditions (i.e. $TS \geq 10\%$)”.

Moreover, in Lines 654-659 the following sentence was added: “The average OLR was 4.5 g VS/kg·d, whereas a maximum 11.5 % TS was reached. In contrast, the addition of beech sawdust to OFMSW allowed to operate co-digestion reactors with an average OLR of 8.3 g VS/kg·d, and reach a maximum 29.0 % TS. Co-digestion lowered by 22 % the TAN content, though an average 186 L/kg VS added of biogas was obtained”.

Finally, in Lines 665-667 the following sentence was added: “Nonetheless, a compromise must be found between increasing the TS content and reducing the specific biogas production by co-digestion, since both aspects strongly determine the HS-AD economy for OFMSW treatment”.

Minor changes, not suggested by the reviewers, were also added throughout the manuscript to enhance its readability and/or reduce the overall length:

In Line 23, “biomass” was substituted by “mass”.

In Lines 25-26, previously stating “the NH₃ inhibition and the rapid solid removal observed prevented to maintain HS-AD conditions” was substituted by “the NH₃ inhibition and the rapid TS removal prevented to maintain HS-AD conditions”.

In Line 27, “Meanwhile” was substituted by “In contrast”.

In Lines 54-55, “potential” was substituted by “risk”.

In Lines 62-63, “among other advantages” was removed.

In Lines 68-71, previously stating “Furthermore, overloading is in many cases related to the presence of methanogenic inhibitors. For example, particular attention must be paid upon the inhibitory effects of NH₃ in HS-AD, due to the high protein content of OFMSW” were substituted by “Furthermore, overloading is in many cases related to the presence of methanogenic inhibitors, such as NH₃, due to the high protein content of OFMSW”.

In Lines 73-75, previously stating “HS-AD is a mature technology, while most of the recently-constructed industrial plants for OFMSW treatment have targeted the semi-continuous HS-AD process” were substituted by “HS-AD of OFMSW is a mature technology, with most of the recently-constructed industrial plants targeting the semi-continuous HS-AD process”.

In Lines 81, “extensively” was removed.

In Lines 88, “encountered” was removed.

In Line 98, “two or three parallel” was substituted by “semi-continuous”.

In Lines 102-103, “biomass retention time” was substituted by “mass retention time (MRT)”.

In Line 104, “these” was substituted by “the”.

In Line 109, “main” was removed.

In Line 122, “working” was substituted by “operated”.

In Line 134, “showed” was substituted by “shown”.

In Line 137, “and/or” was substituted by “and”.

In Line 140-141, previously stating “3.4 kg portions of reactor content were filtered through a 1 mm mesh and used to inoculate co-digestion reactors” were substituted by “3.4 kg of reactor content were filtered through a 1 mm mesh and used to inoculate each co-digestion reactor”.

In Line 176, “and/or” was substituted by “or”, and “supplemented” was substituted by “fed”.

In Line 194, “and/or” was substituted by “or”.

In Lines 207-208, “the MRT was relatively extended in the semi-continuous reactors” was substituted by “the MRT was relatively extended”.

In Line 222, “the free ammonia nitrogen (NH₃)” was substituted by “the NH₃”.

In Lines 231 and 234 “– the” was substituted by “, with an”, while “was” was substituted by “of”.

In Line 247, “with the” was added.

In Line 248, “the” was added.

In Line 250, “Helium” was substituted by “helium”.

In Line 288, “at” was substituted by “on”.

In Lines 290-291, “After two days with no operation (day 20), feeding was resumed in reactor B on day 20, but not in reactor A due to the low pH (i.e. 6.4) observed” was substituted by “After two days with no feed, feeding was resumed in reactor B on day 20, but not in reactor A due to the low pH (i.e. 6.4)”.

In Lines 297 and 355, “Last” was substituted by “The last”.

Lines 315-320, previously stating: “The hydraulic retention time (HRT) and MRT are equivalent concepts defining the average time a fluid and a mass particle, respectively, remain within a continuous reactor. However, the MRT was considered as a more suited indicator in HS-AD, since both the specific weight of the influent/effluent and the reactor mass content might vary, in contrast to ‘wet’ AD were substituted by “In this study, the MRT was considered as a more suited indicator in HS-AD than the hydraulic retention time (HRT), since both the specific weight of the influent/effluent and the reactor mass content varied, in contrast to ‘wet’ AD”

In Line 329, “also” was removed.

In Line 332, “was aimed to promote” was substituted by “promoted”.

In Line 338, “potentially results” was substituted by “resulted”.

In Line 341, “in this study” was removed.

In Line 368, “OLR used” was substituted by “VS fed”.

In Lines 391-393, “The highest TS and VS/TS observed in semi-continuous HS-AD of OFMSW being related to acidification indicate a reduced VS degradation” was substituted by “The highest TS and VS/TS observed in semi-continuous HS-AD of OFMSW were associated with acidification and indicates a reduced VS degradation”.

In Line 403, “might have affected” was substituted by “affects”.

In Line 412, “Further implying” was substituted by “This is a further indication”.

In Line 416, “of” was added.

In Line 429, “Thus, co-digestion” was substituted by “Co-digestion”.

In Line 435, “Moreover” was substituted by “Indeed”.

Paragraph in Lines 447-456 was removed.

In Line 493, “an acetic peak of” was substituted by “a peak of acetic acid”.

In Line 498, “again” was added.

In Line 521, “showed” was substituted by “of OFMSW resulted in”.

In Lines 523-524, “as mentioned in previous subsection” was added.

In Line 525, “of biogas” was added.

In Line 528, “of” was added.

In Lines 531-534, “The CH₄ content reduction is also an indicator of AD imbalance, though it might fail” was substituted by “The reduction of CH₄ content in the biogas is also an indicator of AD imbalance, though it might be inappropriate”.

In Line 536, “cases” was substituted by “reactors”.

In Line 542, “Co-digestion reactor A showed” was substituted by “Co-digestion in reactor A led to”.

In Lines 545-546, “the biogas production” was substituted by “that”.

In Lines 563-564, “(i.e. reactor dilution)” was removed.

In Line 572, “hydrogen sulfide” was substituted by “H₂S”.

In Lines 584-586, “Aiming to recover methanogenesis, water was progressively added to dilute the effect of potential methanogenic inhibitor(s) in all co-digestion reactors.” was substituted by “Aiming to recover methanogenesis in all co-digestion reactors, water was progressively added to dilute the effect of potential methanogenic inhibitor(s)”.

In Line 591, “of” was added, and “were” was substituted by “was”.

In Line 594, “In this study” was substituted by “In conclusion”.

In Lines 596-597, “Therefore” was substituted by “In these conditions”.

In Line 604, “Overloading in this study” was substituted by “In this study, overloading”

In Line 605, “Thus, the” was substituted by “The”.

In Line 616, “The initial TAN in co-digestion” was substituted by “In co-digestion, the initial TAN”.

Finally, “associated to” was substituted by “associated with” in Lines 187, 193, 279, 385, 395-396, 401, 436, 493, 500, 538, 581, 604, 605 and 629.

1 **Semi-continuous Mono-digestion of OFMSW and Co-digestion of**
2 **OFMSW with Beech Sawdust: Assessment of the Maximum**
3 **Operational Total Solid Content**

4

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7

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16

17 **ABSTRACT**

18 In this study, mono-digestion of the organic fraction of municipal solid waste
19 (OFMSW) and co-digestion of OFMSW with beech sawdust, simulating green waste,
20 were used to investigate the maximum operational total solid (TS) content in semi-
21 continuous high-solids anaerobic digestion (HS-AD). To alleviate substrate overloading
22 in HS-AD, the effluent mass was relatively reduced compared to the influent mass,
23 extending the biomass retention time. To this aim, the reactor mass was daily evaluated,
24 permitting to assess the reactor content removal by biogas production. During mono-
25 digestion of OFMSW, the NH₃ inhibition and the rapid ~~TSsolid~~ removal ~~observed~~
26 prevented to maintain HS-AD conditions (i.e. TS ≥ 10 %), without exacerbating the risk
27 of reactor acidification. ~~Meanwhile~~In contrast, the inclusion of sawdust in OFMSW
28 permitted to operate HS-AD up to 30 % TS, before acidification occurred. Therefore,
29 including a lignocellulosic substrate in OFMSW can prevent acidification and stabilize
30 HS-AD at very high TS contents (i.e. 20-30 %).

31

32 **Keywords:** High-Solids Anaerobic Digestion; ~~OFMSW~~; Influent/Effluent Uncoupling;
33 Substrate Overloading; Acidification; Ammonia Inhibition.

34

35 1 INTRODUCTION

36 Anaerobic digestion (AD) of the organic fraction of municipal solid waste (OFMSW),
37 including food waste (FW) and green waste (GW), is a particularly suited treatment
38 biotechnology for energy and by-product recovery (Clarke, 2018; Mata-Álvarez, 2003).
39 ~~(Clarke, 2018; Christensen, 2011; Mata-Álvarez, 2003).~~ In AD, an organic waste is
40 degraded to biogas, mainly composed by CH₄ and CO₂, and a partially stabilized
41 organic digestate, by consortia of different microorganisms working in absence of
42 oxidative species (i.e. O₂ and NO₃⁻) (Astals et al., 2015; Gerardi, 2003). ~~(Gerardi, 2003;~~
43 ~~Switzenbaum et al., 1990).~~

44
45 The sequential steps in AD include hydrolysis, acidogenesis, acetogenesis and
46 methanogenesis, during which different inhibitory substances can be formed leading to
47 inhibitory effects for the anaerobic microorganisms and/or even a complete AD failure.
48 Depending on the concentration, free ammonia (NH₃), hydrogen sulfide (H₂S) and free
49 ions (i.e. Na⁺) are some of the inhibitory substances in AD, affecting predominantly the
50 methanogenic stage, either acetoclastic and/or hydrogenotrophic, and potentially
51 resulting in the buildup of volatile fatty acids (VFA) and H₂ in the system (Astals et al.,
52 2015; Chen et al., 2008). ~~(Chen et al., 2008; Gerardi, 2003; Liu & Boone, 1991; Park &~~
53 ~~Novak, 2013).~~ Meanwhile, the acetoclastic activity results into inorganic carbon (i.e.
54 HCO₃⁻) release in AD, as an important source of pH buffering, minimizing the **potential**
55 **risk** of reactor acidification (i.e. pH ≤ 6.0) by VFA accumulation (Gerardi, 2003).

56
57 The interrelationship between the organic waste characteristics, operational conditions
58 and reactor design determines the AD potential (Karthikeyan & Visvanathan, 2013;

59 Mata-Álvarez, 2003). AD can be differentiated depending on the operational total solid
60 (TS) content into 'wet' (i.e. TS < 10 %) and high-solids AD (HS-AD, i.e. TS ≥ 10 %)
61 (Benbelkacem et al., 2015). HS-AD allows the use of a smaller reactor, reducing the
62 need for water addition and minimizing the digestate production, ~~among other~~
63 ~~advantages~~ (Karthikeyan & Visvanathan, 2013; Pastor-Poquet et al., 2018). However,
64 HS-AD drawbacks include the pervasive chances of reactor acidification due to
65 substrate overload (Benbelkacem et al., 2015). ~~(Benbelkacem et al., 2015; Kayhanian,~~
66 ~~1995).~~ Overloading is the consequence of the slow-growing methanogens being unable
67 to cope with the rapid VFA and/or H₂ buildup resulting from acidogenesis/acetogenesis
68 in HS-AD (Pavan et al., 2000). Furthermore, overloading is in many cases related to the
69 presence of methanogenic inhibitors (Drosg, 2013). ~~For example, particular attention~~
70 ~~must be paid upon the inhibitory effects of, such as NH₃ in HS-AD,~~ due to the high
71 protein content of OFMSW (Kayhanian, 1999).

72

73 HS-AD of OFMSW is a mature technology, ~~with~~ ~~hile~~ most of the recently-constructed
74 industrial plants ~~for OFMSW treatment have targeted targeting~~ the semi-continuous HS-
75 AD process (Mattheeuws, 2016). ~~(De Baere & Mattheeuws, 2013; Mattheeuws, 2016).~~
76 The focus of semi-continuous HS-AD lies on the maximization of the organic loading
77 rate (OLR) that optimizes the methane yield and ensures an adequate organic removal at
78 high TS contents (Benbelkacem et al., 2015; Hartmann & Ahring, 2006). ~~(Benbelkacem~~
79 ~~et al., 2015; Hartmann & Ahring, 2006; Owens & Chynoweth, 1993; Rivard et al.,~~
80 ~~1990).~~ In this line, depending on the organic waste used in HS-AD, the operational TS
81 content is substantially lower than the feed TS, as the organic substrate is ~~extensively~~

82 | converted to biogas by methanogenesis (Pastor-Poquet et al., 2018). ~~(Kayhanian &~~
83 | ~~Hardy, 1994; Mata-Álvarez, 2003)~~
84 |

85 | Therefore, HS-AD lies on a balance between maximizing the OLR and TS content,
86 | while minimizing the chances of reactor failure. Particularly, in order to startup HS-AD,
87 | the OLR needs to be increased relatively slowly, permitting the methanogens to grow
88 | and adapt to the new conditions ~~encountered~~. The transient (non-steady) OLR
89 | modification in HS-AD aims to find an optimum stationary (steady-state) operation to
90 | be used, avoiding acidification and maximizing the economy of the process (Angelidaki
91 | et al., 2006; Bolzonella et al., 2003). However, the risk of inhibition and failure is
92 | undesirably high under HS-AD startup, potentially requiring the implementation of
93 | recovering strategies (i.e. reactor content dilution) to minimize the influence of
94 | inhibitory substances, or even restarting the process when a significant methanogenic
95 | imbalance occurs (Fricke et al., 2007; Kayhanian, 1999).

96 |
97 | This study evaluated the highest tolerable TS content in semi-continuous HS-AD of
98 | OFMSW, by gradually increasing the OLR in ~~two or three parallel~~ semi-continuous
99 | reactors operated at 55°C, until process failure occurred by acidification. Two feeding
100 | strategies were used: mono-digestion of OFMSW and co-digestion of OFMSW and
101 | beech sawdust – as a model lignocellulosic substrate, simulating the inclusion of GW in
102 | OFMSW. Aiming to minimize the risk of substrate overload, the ~~bio~~mass retention time
103 | (MRT) was relatively extended by reducing the effluent compared to the influent mass,
104 | according to the daily mass content removal by biogas production observed in ~~these~~
105 | semi-continuous reactors.

106

107 2 MATERIALS AND METHODS

108 2.1 Substrates and Inoculum

109 The ~~main~~ substrates used in this study were OFMSW and beech sawdust. OFMSW
110 consisted of a mixture of household waste collected in Cassino (Italy), restaurant waste,
111 spent coffee, and garden waste collected at the university facilities, with an
112 approximated wet weight proportion of 45, 35, 15 and 5 % (w/w), respectively.
113 OFMSW was minced twice to a particle size $\leq 5-10$ mm by an industrial mincer
114 [REBER 9500NC, Italy], fully homogenized manually and stored in 5 L buckets at -
115 20°C. During mincing and homogenization, no extra water was added to the raw
116 substrate. A single 5 L bucket of OFMSW was thawed at room temperature overnight,
117 as required to feed the semi-continuous reactors. Goldspan[®] beech sawdust with 1.0-2.8
118 mm particle size was used as co-substrate, to simulate biodegradable
119 green/lignocellulosic waste.

120

121 The inoculum for semi-continuous experiments was obtained from a pre-adapted 'wet'
122 AD (i.e. TS ≤ 5 %) source reactor working-operated at 55°C. The pre-adaptation of 20 L
123 sludge, collected from a mesophilic (35°C) digester treating buffalo manure and
124 mozzarella whey (Capaccio, Italy), consisted of a 4-month progressive feeding of tap-
125 water-diluted OFMSW at 55°C, in order to adapt the inoculum to the new substrate and
126 temperature.

127

128 Prior to start the mono-digestion experiments, the source reactor was kept unfed for 1
129 month and, to consume/reduce the organic content, while continuing with the inoculum

130 | adaptation to the new substrate. Ssubsequently, the feeding with diluted OFMSW was
131 | resumed to recover ~~the~~ methanogenesis ~~activity~~. After 7 and 15 days from the feeding
132 | restart, 4 kg of sludge were taken from the source reactor, filtered through a 1 mm mesh
133 | and used to inoculate the mono-digestion reactors “A” and “B”, respectively. Therefore,
134 | the inoculum was slightly different in reactors A and B, as ~~showed~~ shown in section 3.1.
135 |

136 | During the mono-digestion experiments, the source reactor was periodically fed with
137 | diluted OFMSW and ~~or~~ the mono-digestion reactors effluents, to maintain the reactor
138 | volume and methanogenic activity. Once the mono-digestion experiments ended, the
139 | source reactor was kept unfed for 1 month to serve as inoculum for the co-digestion
140 | experiments. Thus, 3.4 kg ~~portions~~ of reactor content were filtered through a 1 mm
141 | mesh and used to inoculate each co-digestion reactors “A”, “B” and “C”.
142 |

143 | **2.2 Experimental Setup**

144 | The laboratory-scale semi-continuous reactors consisted of 5 L polyethylene
145 | terephthalate (PET) bottles with a modified head allowing the (semi-)solid waste input,
146 | reactor content withdrawal and biogas measurement [Figure 1]. The reactor port was a
147 | polyvinyl chloride (PVC) flexible hosepipe with two valves, easing the reactor
148 | loading/unloading while avoiding air intrusion. The biogas output, containing a
149 | sampling septum, was connected to 5 L Tedlar® bags [Sigma-Aldrich, USA]. All
150 | reactors were maintained at 55°C within a temperature-controlled TCF 400 oven
151 | [ARGOLAB, Italy].

152 |

153 | **2.3 Operation Strategy**

154 Two semi-continuous reactors for mono-digestion of OFMSW or three reactors for co-
155 digestion of OFMSW and sawdust were operated simultaneously in a drag-and-fill
156 mode. The semi-continuous reactors (i.e. kg) and the reactor influents/effluents (i.e. g)
157 were weighed on a ± 0.01 precision scale. ~~The semi-continuous reactors were weighted~~
158 ~~on a ± 0.01 kg precision scale, before/after the discharge/loading operations, while the~~
159 ~~reactor influents/effluents were weighted on a ± 0.01 g precision scale.~~ The OLR was
160 evaluated as the daily ~~VS~~-substrate addition in terms of volatile solids (VS) divided by
161 the reactor mass content (i.e. g VS/kg·d), while the ~~mass retention time (MRT)~~ was
162 evaluated as the quotient between the reactor mass and ~~reactor~~ the daily effluent mass
163 (i.e. days). Since the reactors were fed a maximum of 5 days per week, 7-days moving
164 average OLR and MRT were estimated. Moving-average operational variables are well
165 suited indicators of the immediately preceding operations (i.e. feeding, dilution, reactor
166 content removal) to discern about the risk of VFA buildup in semi-continuous digesters.
167 Moreover, expressing the operational conditions as a moving-average eases the
168 comparison of digesters, when feeding days are not the same or an important mass
169 removal occurs.

170

171 During each drag-and-fill operation, the reactor content was 1) homogenized before
172 opening the system, 2) sampled and 3) analyzed mainly for pH and alkalinity – since pH
173 had to be maintained over 6.5, as an important methanogenic inhibition might take place
174 below this threshold (De Vrieze et al., 2012; Gerardi, 2003). ~~(Gerardi, 2003;~~
175 ~~Switzenbaum et al., 1990)~~ Depending on the pH and alkalinity, 4) the proper amount of
176 substrate was used ~~and~~/or diluted as needed, 5) prior to be supplemented fed to the

177 reactors. Finally, 6) the reactor content was homogenized once again, while the Tedlar®
178 bags were checked for biogas production and subsequently emptied.

179

180 To increase the reactor TS content from ‘wet’ AD (i.e. TS < 5 %) to HS-AD (i.e. TS ≥

181 10 %), the OLR was controlled by increasing/decreasing the daily amount of substrate

182 and/or tap water addition based on the methanogenic activity, and aiming to minimize

183 the substrate overload. To evaluate the differences in the reactor performance, m Mono-

184 ~~co~~-digestion reactors were fed in parallel ~~though using~~ different OLR/MRT ~~were~~

185 ~~used in each reactor~~, as shown in section 3.2. Subsequently, co-digestion reactors were

186 also operated in parallel at three different OLR/MRT. In each reactor, tThe

187 methanogenic activity was roughly associated ~~to~~ with the relative increase of the pH and

188 inorganic carbon alkalinity (ALK_p), the reduction of the reactor mass content and the

189 biogas production compared to previous operational values, as also mentioned in

190 section 3.2. For example, a relative pH and ALK_p increase of approximately 0.5 pH

191 units and 0.3 g CaCO₃/kg, respectively, alongside a reactor mass removal of about 30-

192 50 g/d and a specific biogas production higher than 250 mL/kg ~~Reactor-reactor~~

193 ~~Content~~ ~~content~~ ~~d~~ were associated ~~to~~ with ongoing methanogenesis, indicating that the

194 OLR could be maintained ~~and/or~~ relatively increased. Similarly, the relative increase of

195 intermediate alkalinity (ALK_i) (i.e. 0.5 g Acetic Acid/kg) was used as a preliminary

196 indicator of the potential VFA buildup and risk of substrate overload (Lahav et al.,

197 2002).

198

199 All these parameters were further complemented with the user’s evaluation of the

200 previous operation, in order to decide for the daily feed/dilution to be used. Thus, all

201 reactors were started with a low OLR (i.e. 2 g VS/kg·d) that was gradually increased to
202 increase the TS content. As reactor performance deteriorated with increasing OLR, the
203 reactor feeding was reduced/stopped to prevent acidification (i.e. $\text{pH} \leq 6.0$).

204

205 The reactor mass was maintained constant by reducing the effluent compared to the
206 influent mass, according to the observed reactor mass content removed by biogas
207 production from the previous operation. With this strategy, the MRT was relatively
208 extended ~~in the semi-continuous reactors~~, aiming to promote the methanogenic
209 adaptation in case of overloading. Semi-continuous reactors were fed until acidification
210 occurred. From this point, feeding was stopped and reactor dilution and/or inorganic salt
211 addition (i.e. NaHCO_3 and FeCl_2) were tested as recovering strategies. A summary of
212 the weekly operational variables is presented ~~as Supplementary Information in Table 1.~~

213

214 **2.4 Bio-Physical-Chemical Analyses**

215 ~~The pH, TS, volatile solids (VS), total Kjeldahl (TKN) and ammonia (TAN) nitrogen,~~
216 ~~and the total hydrogen sulfide were determined by the standard methods (APHA, 1999).~~
217 ~~The pH, The partial-(ALK_p) and intermediate-(ALK_i) alkalinity were determined from~~
218 ~~the supernatant of solid and semi-solid samples as proposed by (Lahav et al., 2002),~~
219 ~~after diluting the sample with distilled water, homogenization and centrifugation at~~
220 ~~6000 rpm for 15 min (EPA, 2015). The TS and VS content, total Kjeldahl (TKN) and~~
221 ~~ammonia (TAN) nitrogen, and the total H_2S were determined by the standard methods~~
222 ~~(APHA, 1999). The free ammonia nitrogen (NH_3) was approximated as a function of~~
223 ~~TAN and pH, as shown by (Astals et al., 2015). (Angelidaki & Ahring, 1993).~~ The VFA
224 (acetic, propionic, butyric and valeric acids) were measured with an LC-20AD HPLC

225 [Shimadzu, Japan], mounting a Rezex ROA-Organic Acids 8+ column coupled to a 210
226 nm UV detector, and using 0.0065 M H₂SO₄ at 0.6 mL/min as mobile phase. The biogas
227 composition (CH₄, CO₂ and H₂) was analyzed with a 3400 GC-TCD [Varian, USA],
228 using argon as carrier gas.

229

230 The biomethane potential (BMP) test for OFMSW used 3.0 g of substrate, 50.0 g of
231 source inoculum, 40.0 g of distilled water and 0.10 g of NaHCO₃ in 280 mL bottles (6
232 replicates)—~~the~~, with an inoculum-to-substrate ratio (ISR) ~~was of~~ 2.0 g VS/g VS. The
233 BMP test for sawdust used 1.0 g substrate and 50.0 g of inoculum in 160 mL bottles (3
234 replicates)—~~the~~, with an ~~the~~ ISR ~~was of~~ 1.0 g VS/g VS. BMP tests were performed
235 according to Angelidaki and Sanders (2004) and Holliger et al. (2016). In the BMP test
236 for OFMSW, the distilled water and NaHCO₃ addition served to minimize the chances
237 of inhibition (i.e. by NH₃) and acidification, respectively. In contrast, NH₃ build-up and
238 acidification were not expected in the BMP test of sawdust, due to the low nitrogen
239 content and the reduced biodegradability of sawdust, as thoroughly discussed in next
240 section, permitting also to use a lower ISR. Both BMP tests lasted longer than 100 days.
241 Blank assays included the inoculum and further distilled water compensating for the
242 absence of substrate, using three replicates in each BMP. Inoculum activity assays using
243 a reference substrate were not performed.

244

245 The BMP was the normalized methane production (P = 1 bar, T = 0°C), excluding the
246 methane production of the inoculum, per unit of substrate VS added. The gas production
247 was evaluated with a two-vessel displacement system, with the first vessel containing 4
248 N NaOH to capture CO₂ and the second vessel containing water to be ‘displaced’. The

249 bottles were sealed with butyl rubber stoppers and aluminum crimps and flushed with
250 ~~Helium~~helium, before adding 0.2 mL of 10 g/L Na₂S piercing the septum to ensure an
251 adequate redox potential (Angelidaki & Sanders, 2004). All bottles were incubated at
252 55°C and agitated only while measuring the gas production.

253

254 2.5 Statistical Analyses

255 The Dixon's test for BMP outliers was applied as recommended by Holliger et al.
256 (2016). The unpaired t-test of Microsoft Excel 2016 (Microsoft, USA) was applied to
257 determine the statistical significance of experimental data, using the two-tail p-value at
258 95 % confidence.

259

260

261 **3 RESULTS AND DISCUSSION**

262 **3.1 Bio-Physical-Chemical Characterization of Substrates and Inoculum**

263 OFMSW showed a TS of 26 %, a VS/TS ratio of 0.93 and a TKN of 24.8 g N/kg TS, in
264 agreement with real source-sorted OFMSW (Angelidaki et al., 2006; Bolzonella et al.,
265 2006; Jokela & Rintala, 2003). ~~(Cecchi et al., 2002; Jokela & Rintala, 2003).~~ The high
266 VS/TS ratio of OFMSW (i.e. > 0.9) indicated minimal presence of inert materials
267 (Pavan et al., 2000). Sawdust showed a TS of 94 % and a VS/TS ratio of 0.99, similar to
268 those obtained by Brown and Li (2013) for 40°C-dried yard waste, suggesting that
269 beech sawdust could simulate GW. The BMP of OFMSW and sawdust was ~~497457~~ and
270 ~~148-161~~ NmL CH₄/g VS, respectively. Table ~~2-1~~ shows the bio-physical-chemical
271 characterization of OFMSW and sawdust. Despite the thorough mincing and
272 homogenization, Minor-minor modifications were observed in the OFMSW

273 | characterization (i.e. TS, TKN or BMP), were mainly attributed to the substrate
274 | heterogeneity.

275

276 | The inoculum in mono-digestion reactors A and B showed a common TS and TKN of
277 | 2.8 % and 161 g N/kg TS, respectively. An initial acetic acid concentration of 2.300 and
278 | 3.300 mg/kg was observed in reactors A and B, respectively, being this difference
279 | associated ~~to~~ with the later inoculation of reactor B than reactor A. The inoculum used
280 | in co-digestion reactors showed a TS of 2.5 %, a TKN of 139 g N/kg TS and an acetic
281 | acid concentration of 0.020 mg/kg. The inoculum compositions are shown in Table 32.

282

283 | 3.2 Semi-continuous Operation – Increasing the TS Content

284 | 3.2.1 Mono-digestion of OFMSW

285 | Mono-digestion results are summarized in Figures 2 and 3. The weekly-averaged results
286 | were also included as Supplementary Information. The 7-days average OLR in reactors
287 | A and B was varied from an initial 2.4 (day 6) and 6.0 (day 13) g VS/kg·d to 4.9 and 5.5
288 | g VS/kg·d, respectively, at-on day 17 [Figure 2a]. Thus, a common OLR (i.e. around 5 g
289 | VS/kg·d) was achieved, aiming to compensate for the 1-week-lagged inoculation in
290 | reactor B. After two days with no ~~operation-feed(day-20)~~, feeding was resumed in
291 | reactor B on day 20, but not in reactor A due to the low pH (i.e. 6.4) ~~observed~~ [Figure
292 | 3a]. As pH recovered in reactor A (i.e. from 6.4 on day 21, to pH=7.6, on day 29) due
293 | to methanogenesis activity, feeding was ~~also~~ resumed. During the same period, ALK_p in
294 | reactor A increased alongside pH from 1.1 to 2.7 g CaCO₃/kg (data not shown), as an
295 | indicator of ongoing methanogenesis. By day 45, a maximum OLR of 6.8 and 8.5 g
296 | VS/kg·d was reached in reactors A and B, respectively. After day 48, the OLR required

297 | progressive reduction to minimize the risk of acidification. ~~The H~~ast feeding in reactors
298 | A and B was implemented on days 78 and 73, respectively, as both reactors showed pH
299 | ≤ 6.5 and CH_4 content $\leq 40\%$ [Figure 2f]. From this point, mono-digestion reactors
300 | were left unfed aiming to promote the recovery of methanogenesis.

301 |
302 | The OLR in reactor B was averagely about 1.5 g VS/kg·d higher than that used in
303 | reactor A during the whole experiment ($p < 0.001$), explaining the relatively faster
304 | acidification observed in reactor B. Thus, prior to the occurrence of acidification,
305 | reactor B was fed with a ~~daily n average 20-35~~ g VS/d, significantly higher compared
306 | ~~to~~ than the ~~15-26~~ g VS/d used for reactor A ($p = 0.03$). The initial MRT was 55 (day 6)
307 | and 29 days (day 13) for reactors A and B, respectively, and was gradually increased to
308 | maintain the methanogenic performance at higher OLR [Figure 2b]. Noteworthy, the
309 | MRT and OLR in these semi-continuous reactors did not show an inverse pattern, since
310 | the dilution as well as the influent and effluent mass flows used were different to
311 | account for the organic removal.

312 |
313 | Uncoupling the influent and effluent mass flows in the semi-continuous reactors, based
314 | on the HS-AD reactor content removal by methanogenesis, permitted to increase the
315 | MRT and OLR simultaneously. ~~The hydraulic retention time (HRT) and MRT are~~
316 | ~~equivalent concepts defining the average time a fluid and a mass particle, respectively,~~
317 | ~~remain within a continuous reactor. However, In this study,~~ the MRT was considered as
318 | a more suited indicator in HS-AD than the hydraulic retention time (HRT), since both
319 | the specific weight of the influent/effluent and the reactor mass content might
320 | vary/varied, in contrast to ‘wet’ AD (Pastor-Poquet et al., 2018). ~~(Rivard et al., 1990).~~

321 ~~As an example, the occurrence of methanogenesis led to a 60 g removal of the reactor~~
322 ~~mass content in both mono-digestion reactors from day 37 to 41 (data not shown). Thus,~~
323 ~~Prior to the occurrence of reactor acidification,~~ the weekly effluent mass was ~~1-38~~
324 ~~%significantly~~ higher than the influent ~~(i.e. 18 %; p = 0.03)~~ to maintain the mono-
325 digestion reactors mass content constant. ~~[Table 1], with the exception of weeks 5 and~~
326 ~~12 in reactor A, when a reduced influent was used to prevent acidification but the~~
327 ~~reactor was sampled for physical-chemical analyses.~~

328

329 The MRT-uncoupling concept was proposed by Richards et al. (1991) and was ~~also~~
330 used by Kayhanian and Rich (1995) to operate a pilot-scale semi-continuous HS-AD
331 reactor fed with OFMSW. In this study, uncoupling the influent and effluent in HS-AD
332 ~~was aimed to~~ promoted the methanogenic adaptation to overloading conditions and/or
333 the buildup of inhibitors (i.e. NH₃) during the OFMSW degradation. Noteworthy, the
334 MRT must be longer than the doubling time of methanogens (i.e. 20-30 days) to avoid
335 their ‘washout’ from continuous HS-AD reactors, while the methanogenic doubling
336 time might lengthen considerably in presence of inhibitory substances (i.e. NH₃)
337 (Drosg, 2013; Gerardi, 2003; Rittman & McCarty, 2001). Therefore, extending the
338 MRT ~~potentially results~~ resulted in a more stable HS-AD operation (Hartmann &
339 Ahring, 2006; Rajagopal et al., 2013). ~~(Climenhaga & Banks, 2008; Hartmann &~~
340 ~~Ahring, 2006; Rajagopal et al., 2013)~~, though the sole implementation of influent-
341 effluent uncoupling ~~in this study~~ was not sufficient to avoid HS-AD overloading and
342 acidification during mono-digestion of OFMSW.

343

344 **3.2.2 Co-digestion of OFMSW and Sawdust**

345 Co-digestion results are summarized in Figures 4 and 5. The 7-days average OLR was
 346 increased from 4.5-4.9 g VS/kg·d (day 6) up to 10.9, 12.1 and 12.6 g VS/kg·d (day 23)
 347 in reactors A, B and C, respectively. To avoid acidification, feeding was stopped in
 348 reactors A and B from day 26, while the OLR was only reduced to 5.0 g VS/kg·d in
 349 reactor C [Figure 4a]. As pH recovered (i.e. ≥ 7.0) [Figure 5], feeding was resumed in
 350 reactors A and B. A maximum OLR of 14.8 g VS/kg·d was reached in reactor C (day
 351 47) using a sawdust/OFMSW ratio of 2.1 g VS/g VS, prior to the occurrence of reactor
 352 acidification (day 56). The maximum OLR in reactor B was 15.1 g VS/kg·d (day 55)
 353 using a sawdust/OFMSW ratio of 1.6 g VS/g VS, while an OLR of 16.0 g VS/kg·d was
 354 reached in reactor A during the same period, using a sawdust/OFMSW ratio of 1.3 g
 355 VS/g VS. ~~The last~~ feeding in reactors A and B was performed on day 76, as a slight
 356 but continued drop in pH [Figure 5] and CH₄ [Figure 4f] was observed in both reactors.

357

358 The ~~maximum average~~-OLR used for co-digestion was two times higher than ~~that~~ for
 359 mono-digestion (i.e. ~~8.3-13.0~~ vs. ~~6.5-4.5~~ g VS/kg·d, respectively; $p < 0.001$), due to the
 360 lower biodegradability of sawdust, though the ~~maximum~~-OLR only due to OFMSW was
 361 similar in both cases (i.e. ~~7.5-4.1~~ vs. ~~4.5~~ g VS/kg·d, ~~respectively~~; $p = 0.07$). Thus, a
 362 ~~common maximum~~ OLR of 7.5-8.0 g VS/kg·d related to the sole supplementation of
 363 OFMSW was ~~reached-used~~ in the three co-digestion reactors on day 21, while the OLR
 364 solely due to OFMSW was subsequently maintained below 6.0 g VS/kg·d, as sawdust
 365 was increased in the feeding mixture [Figure 4a]. In terms of average VS fed, reactor C
 366 was operated under ~~relatively~~ more stressing feeding conditions than reactors A and B
 367 (i.e. ~~3553; 11 and~~ vs. ~~14-44~~ g VS/d, respectively; $p = 0.15$), being again the fastest
 368 occurrence of reactor acidification related to the highest ~~OLR-used~~ VS fed.

369

370 The initial MRT was higher than 168 days (day 6) and was decreased to 30 days (day
371 17), similarly in the three reactors [Figure 4c]. From this point, the MRT reached an
372 average of 85 days (day 35) and was subsequently reduced to an average of 37 days
373 (day 53) in all reactors, before being progressively increased to minimize the substrate
374 overload. The MRT was relatively-significantly lower in co-digestion than mono-
375 digestion (i.e. 69 vs. 92 days, respectively; $p < 0.001$), as lower MRT were
376 predominantly linked to the higher OLR used in co-digestion.

377

378 **3.3 Influence of the Substrate Composition on the TS Increase**

379 The OLR/MRT control in the mono-digestion reactors fed with OFMSW permitted to
380 increase the TS content, balancing the VFA accumulation with the rapid organic
381 degradation observed [Figures 2 and 3]. Reactors A and B were started at TS = 2.8 %
382 and reached a maximum of 10.7 (day 79) and 11.7 % (day 69), respectively [Figure 2c],
383 being these TS slightly higher than the lower HS-AD threshold (i.e. $TS \geq 10$ %). The
384 highest TS in the semi-continuous reactors did not coincide with the maximum OLR,
385 but were predominantly associated ~~to~~with low pH (i.e. ≤ 6.5), when methanogenesis
386 was potentially inhibited. In this line, a gradual increase of the VS/TS ratio (data not
387 shown) was observed in both reactors from 0.69 (day 0) to 0.82 (day 40), reaching a
388 maximum value of 0.87, prior acidification occurred on days 79 and 76 in reactors A
389 and B, respectively.

390

391 The highest TS and VS/TS observed in semi-continuous HS-AD of OFMSW being
392 related to acidification~~were associated with acidification and~~ indicates a reduced VS

393 degradation alongside inhibitory conditions. Particularly, the lowest HS-AD threshold
394 (i.e. TS = 10 %) using OFMSW was reached only under extreme overloading. A more
395 stable HS-AD fed with an easily biodegradable OFMSW (i.e. FW) is also associated ~~to~~
396 with a TS increase alongside overloading/inhibitory conditions. For example, Tampio et
397 al. (2014) reported a TS increase from 7 to 8 % during 400 days of semi-continuous AD
398 fed with FW, though TS rapidly reached 11 % during the next 50 days of operation,
399 when reactor inhibition was likely occurring. In the same line, Bolzonella et al. (2003)
400 reported a TS increase from 5 to 15 % during the initial 60 days of continuous AD pilot-
401 scale startup fed with OFMSW, being the maximum TS associated ~~to~~with the highest
402 total VFA observed (i.e. 2.8 g Acetic Acid/L). All these results were likely related to
403 methanogenesis inhibition, since the VFA accumulation ~~might have affected~~affects the
404 hydrolysis/acidogenesis rates, hampering the organic removal in HS-AD (Vavilin et al.,
405 2008).

406
407 The maximum TS obtained in this study for semi-continuous HS-AD of OFMSW
408 should be considered as indicative (only) of those obtainable in steady-state digesters,
409 since the transient/acidification conditions potentially reduced the VS removal. Thus,
410 the operational TS content of stable digesters fed with the easily biodegradable content
411 of OFMSW (i.e. FW) might be lower than those observed along non-steady-state
412 conditions. ~~Further implying~~This is a further indication that a steady-state semi-
413 continuous reactor using an easily biodegradable OFMSW as a substrate might not be
414 operated within the HS-AD threshold (i.e. $TS \geq 10\%$).

415

416 Co-digestion permitted to increase TS from 2.5 % (day 0) up to a maximum of 33.2
417 (day 79), 26.7 (day 76) and 27.0 % (day 57) in reactors s A, B and C, respectively [Figure
418 4c]. Hence, the maximum TS reached in co-digestion before the reactors acidified (i.e.
419 29.0 ± 2.8 %) was considerably higher than the lower HS-AD threshold (i.e. TS ≥ 10 %) and the maximum TS of mono-digestion (i.e. 11.5 ± 0.5 %), due to the addition of
420 sawdust to OFMSW. The highest TS was related again to acidified (i.e. pH < 6.5) or
421 acidifying (i.e. downward trend on pH/CH₄ content) conditions, as observed for mono-
422 digestion. Thus, the VS/TS ratio in co-digestion (data not shown) increased from 0.65
423 (day 0) to 0.90 (day 40), reaching a maximum of 0.95 before reactors acidified (day 76),
424 due to both the higher VS/TS ratio of sawdust and the reduced VS removal during
425 inhibitory conditions.
426

427

428 These results showed that the particular characteristics of OFMSW determined the
429 maximum operating TS content in semi-continuous HS-AD. ~~Thus, Co-~~digestion of
430 OFMSW and sawdust resulted in a approximately three times higher TS than mono-
431 digestion (i.e. ~~33-29.0~~ and 11.5 %, respectively). The inclusion of sawdust in OFMSW
432 favored the rapid TS and OLR increase compared to mono-digestion due to the higher
433 TS and the lower biodegradability of sawdust, as demonstrated by the substantially
434 lower BMP of sawdust than that of OFMSW (i.e. ~~148-161~~ and ~~457-497~~ NmL CH₄/g VS,
435 respectively) [Table 21]. ~~Moreover~~Indeed, lignocellulosic materials (i.e. GW) are
436 normally associated ~~to~~ with a reduced biodegradation rate, compared to more easily
437 degradable substrates (i.e. FW), due to the high lignin content hampering hydrolysis
438 (Brown & Li, 2013; Mancini et al., 2018a; Vavilin et al., 2008). ~~(Brown et al., 2012;~~
439 ~~Chavez Vazquez & Bagley, 2002; Mancini et al., 2018a)~~, being also beneficial to limit

440 the VFA buildup in HS-AD. On the other hand, TAN was ~~approximately 30-22~~ % lower
441 ~~during~~ co-digestion than mono-digestion ~~(i.e. 2.9 vs. 3.7 g N/kg, respectively; p <~~
442 ~~0.001)~~ [Figures 2 and 4], due to the lower TKN of sawdust [Table 21]. Noteworthy, the
443 TAN accumulation was likely promoting methanogenic inhibition in this study, as
444 further discussed in section 3.5. Therefore, using sawdust – as GW – was also adequate
445 to adjust the carbon-to-nitrogen (C/N) ratio in HS-AD of OFMSW.

446

~~Introducing a lignocellulosic substrate in OFMSW is a well suited strategy to operate
447 semi-continuous reactors in HS-AD conditions (i.e. 20-30 % TS), since the maximum
448 operational OLR and TS in semi-continuous HS-AD of OFMSW is determined by the
449 substrate characteristics (i.e. TS, VS/TS ratio and biodegradability) and/or the buildup
450 of inhibitors (i.e. NH₃) (Bolzonella et al., 2006; Kayhanian & Hardy, 1994; Mata-
451 Alvarez et al., 2014; Pavan et al., 2000; Rivard et al., 1990). Meanwhile, the TS content
452 is further determined by the organic removal, since a TS removal ranging from 30 to 80
453 % has been reported in semi-continuous HS-AD of OFMSW, depending on the
454 substrate characteristics, but also reactor design and operation (Bolzonella et al., 2006;
455 Mata-Álvarez, 2003; Pavan et al., 2000)~~

457

458 3.4 Main Indicators of Substrate Overload

459 3.4.1 Evolution of pH and VFA

~~The start up of biogas plants requires a gradual and slow OLR increase to adapt
460 progressively the methanogens, avoiding VFA accumulation in the digester.
461 (Angelidaki et al., 2006; Drog, 2013; Marchaim & Krause, 1993). However, all the
462 semi-continuous reactors in this study had to be operated under overloading conditions,
463~~

464 ~~to increase TS from 'wet' AD to HS AD. Thus, acidification was expected due to the~~
465 ~~high OLR used, but also because acidification is likely to occur in HS AD due to the~~
466 ~~high organic content in the reactor (Kayhanian, 1995). Particularly, pH fluctuated as a~~
467 ~~result of the OLR modification and the activity of microorganisms, showing a~~
468 ~~downward trend associated to the VFA accumulation, until a sharp acetic acid buildup~~
469 ~~occurred. This rapid acetic acid buildup indicated a significant inhibition of acetoclastic~~
470 ~~methanogenesis, subsequently resulting in reactor acidification (i.e. $\text{pH} \leq 6.0$).~~

471

472 pH in mono-digestion reactor A decreased from 8.1 to 6.4 due to the rapid acetic acid
473 buildup (i.e. from 3.000 to 9.000 mg/kg) observed during the initial 20 days of
474 operation [Figure 3a]. As feeding was stopped from day 20 to 29, pH reached 7.6, while
475 acetic acid decreased below 0.700 mg/kg right afterwards (day 34). Propionic, butyric
476 and valeric acids gradually increased from < 0.150 mg/kg (day 0) to 5.000 , 4.000 and
477 1.100 mg/kg (day 79), respectively. From day 79, pH dropped from 7.1 to 6.1, linked to
478 a sudden acetic acid increase from 3.000 to 5.000 mg/kg, and the subsequent CH_4
479 content drop from 56 to 37 % [Figure 2f].

480

481 Mono-digestion reactor B was relatively more stressed than reactor A, as indicated by
482 the wider acetic acid fluctuations (i.e. ± 4.000 mg/kg) and the rapid accumulation of
483 propionic acid from 1.200 (day 7) to 5.800 mg/kg (day 73) [Figure 3b]. The VFA
484 fluctuation is in line with the fact that methanogens grow relatively slower than the
485 hydrolytic/acidogenic microorganisms in AD (De Vrieze et al., 2012; Gerardi, 2003).
486 ~~(Gerardi, 2003; Marchaim & Krause, 1993).~~ Thus, the higher OLR used in reactor B led
487 to a more pronounced methanogenic/acidogenic imbalance, exacerbating the VFA

488 accumulation. The VFA buildup led to a pH decrease from 8.4 to 6.2 in reactor B during
489 the whole experimental period, while a significant acetoclastic inhibition occurred from
490 day 70 to 73, when acetic acid abruptly increased from 2.700 to 5.800 mg/kg.

491

492 pH in co-digestion reactor A gradually decreased from 8.7 to 6.4 along the experimental

493 period, showing a minimum of 6.1 associated ~~to-with an acetic~~ peak of acetic acid of
494 8.300 mg/kg (day 26) [Figure 5a]. Acetic acid was considerably consumed (i.e. < 0.360

495 mg/kg) by day 47 due to ongoing methanogenesis, and progressively increased

496 thereafter by overloading. Similarly, pH in reactor B showed a minimum of 6.3 when

497 acetic acid peaked at 8.200 mg/kg (day 26) [Figure 5b], while the acetic acid was

498 extensively consumed (i.e. < 0.350 mg/kg) by day 41 prior to increase again steadily. In

499 reactor C, acetic acid had a similar evolution with a maximum of 7.200 mg/kg (day 26)

500 [Figure 5c], while pH dropped to 6.0 on day 57, associated ~~to-with~~ a sharp acetic acid

501 build-up from 1.000 to 3.700 mg/kg. Propionic, butyric and valeric acids increased from

502 0.500, 0.140 and 0.005 mg/kg (day 0) to a maximum range of 3.000-3.500, 2.900-3.200

503 and 2.500-2.600 mg/kg, respectively, obtained right after acidification occurred on day

504 79 in reactors A and B, and on day 56 in reactor C. The pH was relatively lower (i.e. 2

505 %; p = 0.13) and total VFA was relatively higher (i.e. 5 %; p = 0.25) profiles showed

506 wider fluctuations during mono-digestion than co-digestion, likely due to the faster

507 degradation rates but also the higher release of inhibitory compounds related to

508 OFMSW than sawdust ~~inhibitory content of OFMSW compared to sawdust~~, as discussed

509 before in section 3.3.

510

511 Feeding the reactors a maximum of 5 days per week influenced the reactor dynamics,
512 since pH increased and VFA – mainly acetic acid – decreased during the periods with
513 no feed. The pH and VFA modifications [Figures 3 and 5] were associated with the TS
514 removal, as mentioned before, and also affected the biogas production/composition, and
515 the TAN buildup [Figures 2 and 4]. As an example, in co-digestion reactor A, pH
516 increased from 7.0 to 7.9 from day 37 to 41, while acetic acid decreased from 4.30 to
517 2.40 g/kg, triggering a biogas production of 0.6 L/kg reactor content and a methane
518 content increase from 59 to 70 % [Figure 4 and 5].

519

520 **3.4.2 Biogas Production and Composition**

521 Mono-digestion ~~showed of~~ OFMSW resulted in a cumulative biogas production of 65
522 and 66 L/kg ~~Reactor-reactor Content-content~~ in reactor A and B, respectively [Figure
523 2e]. Biogas production was mainly correlated to the acetic acid consumption [Figure
524 3a], as mentioned in the previous subsection. For example, 21 L/kg ~~Reactor-reactor~~
525 Content-content of biogas were measured during the initial 20 days of reactor A, before
526 acetic acid accumulated and biogas production slowed down.

527

528 Biogas composition measurements started on day 60 showing an average of 63 % CH₄
529 in both mono-digestion reactors [Figure 2f], which subsequently fluctuated showing a
530 downward trend alongside the VFA accumulation. The CH₄ content dropped below 40
531 % in both reactors right after biogas production definitely ceased on days 78-79. The
532 reduction of CH₄ content ~~reduction in the biogas~~ is also an indicator of AD imbalance,
533 though it might ~~fail be inappropriate~~ to assess rapid changes in the reactor performance
534 (Drosg, 2013). The highest H₂ concentration (data not shown) was 1.8 and 1.1 % on day

535 | 59 in reactors A and B, respectively, while H₂ remained below 0.8 % in both
 536 | ~~cases reactors~~, during the rest of the experiment. The presence of H₂ indicated that the
 537 | hydrogenotrophic methanogens were unable to cope with the rapid H₂ production from
 538 | acidogenesis, since H₂ higher than 1-2 % in the gas phase is normally associated ~~to with~~
 539 | AD overloading (Drosg, 2013; Molina et al., 2009). ~~(Drosg, 2013; Marchaim & Krause,~~
 540 | ~~1993; Switzenbaum et al., 1990).~~

541

542 | Co-digestion in reactor A ~~showed~~ led to a cumulative biogas production of 48 L/kg
 543 | ~~Reactor reactor Content content~~, while 49 and 27 L/kg ~~Reactor reactor Content content~~
 544 | were observed in reactors B and C, respectively [Figure 4e]. In spite of the higher OLR
 545 | used in co-digestion, the biogas production was considerably lower than ~~the biogas~~
 546 | ~~production that~~ obtained with mono-digestion (i.e. 65 L/kg ~~Reactor reactor~~
 547 | ~~Content content~~). Thus, the specific biogas production was ~~166-192229 ± 20~~ L/kg VS
 548 | ~~added Fed~~ in mono-digestion and ~~59-7186 ± 18~~ L/kg VS ~~added Fed~~ in co-digestion (i.e.
 549 | 62 % lower), due to the reduced biodegradability of sawdust.

550

551 | The CH₄ content [Figure 4f] reached a peak of 75 % during the first two weeks of
 552 | operation in the three co-digestion reactors, but it decreased subsequently as VFA
 553 | accumulated [Figure 5]. A minimum 43 % CH₄ was detected in reactor A associated
 554 | with the last biogas production observed (day 82), while a sharp drop from 60 to 29 %
 555 | CH₄ was observed in reactor C right after day 60. H₂ was detected at 0.3 % in the three
 556 | co-digestion reactors on day 23 (data not shown). Thereafter, H₂ was not detected in
 557 | reactor A, while reactor B showed a single H₂ peak of 1.5 % on day 70, right after the
 558 | reactor was accidentally opened to the atmosphere. In reactor C, H₂ peaks of 1.7, 1.2

559 and 1.6 % were observed on days 41, 47 and 58, respectively, supporting the occurrence
560 of a more extensive overload in this reactor.

561

562 3.5 Testing Recovering Strategies

563 Once acidification occurred, feeding was stopped and some recovering strategies (~~i.e.~~
564 ~~reactor dilution~~) were tested to resume methanogenesis. In mono-digestion reactor A, a
565 3 M NaHCO₃ buffer solution was added on days 83 and 84 to raise the pH (i.e. from 6.2
566 to 6.8), ~~and return the pH~~ within a suitable range for methanogens (i.e. 6.5-7.0). Adding
567 NaHCO₃ is normally used to counteract acidification when digesters show a reduced
568 ALK_p (Chen et al., 2008; Holliger et al., 2016). However, methanogenesis did not
569 recover after more than 20 days.

570

571 On day 76, FeCl₂ was supplemented to mono-digestion reactor B in a higher amount
572 than the stoichiometric, to precipitate the total ~~hydrogen sulfide~~ H₂S in the system (i.e.
573 30 mg H₂S/kg, data not shown). However, FeCl₂ overdosing resulted in a pH drop from
574 6.3 to 5.7 (days 76-77), ~~and~~ Thus, 2 M NaHCO₃ solution was rapidly added to recover
575 the pH to 6.6 (day 77). Both Fe²⁺ and/or Fe³⁺ can be used to precipitate sulfide in AD,
576 but Fe²⁺ was preferred in this study to avoid the inclusion of a strong electron acceptor
577 (i.e. Fe³⁺) that could react with organic compounds in the anaerobic digester (i.e. Fe³⁺ +
578 1/2 H₂ → Fe²⁺ + H⁺, ΔG^o << 0) (Fermoso et al., 2015; Rittman & McCarty, 2001). After
579 2 weeks of methanogenic inhibition (day 90), 200 g of 'wet' AD inoculum from the
580 source reactor were added to reactor B, allowing a gradual methanogenic recovery,
581 associated ~~to~~ with an increase of pH from 6.9 to 7.3 [Figure 3b] and CH₄ content from
582 20 to 52 % [Figure 2f], until the end of the reactor operation.

583

584 | Aiming to recover methanogenesis in all co-digestion reactors, water was progressively
585 | added to dilute the effect of potential methanogenic inhibitor(s) ~~in all co-digestion~~
586 | ~~reactors~~. The progressive addition of low amounts of water in co-digestion reactors
587 | permitted to maintain HS-AD conditions (i.e. $TS \geq 10\%$), thanks to the elevated TS
588 | content reached before reactors acidified (i.e. $TS \geq 30\%$). Dilution was performed in
589 | reactors A and B from day 79 and in reactor C from day 62. In reactor A and B an
590 | average of 180 and 170 mL of water was used, respectively, on days 79, 82, 84, 91 and
591 | 98, while an average of 160 mL of water ~~were was~~ added to reactor C on days 62, 63,
592 | 68 and 91.

593

594 | In ~~this study~~conclusion, neither water, nor buffer addition permitted to recover
595 | acidified/acidifying HS-AD reactors, probably because of the important imbalance
596 | between methanogens and acid-producers in the system (Gerardi, 2003). ~~Therefore~~In
597 | these conditions, inoculum addition might be the only way to recover an acidified HS-
598 | AD reactor, though emptying and re-inoculating the reactor might be necessary (Fricke
599 | et al., 2007). Another strategy to prevent reactor acidification and/or enhance the
600 | digester performance is trace element (i.e. Se, Ni, Co, Mo, W) addition (Fermoso et al.,
601 | 2015; Mancini et al., 2018b). However, this was out of the scope of this manuscript.

602

603 | **3.6 Ammonia Buildup**

604 | In this study, overloading ~~in this study~~ was associated ~~to with~~ the high OLR used, but
605 | also ~~to with~~ the NH_3 buildup, in the semi-continuous reactors. ~~Thus,~~ the high OLR and
606 | the degradation of the protein content of OFMSW increased the TAN content in both

607 mono-digestion reactors [Figure 2d]. TAN ranged from 3.4 g N/kg (day 0) to a
608 maximum of 4.8 and 4.9 g N/kg in reactors A and B (day 104), respectively, with both
609 reactors showing a minimum TAN of 3.0 g N/kg around day 20. The initial NH₃ was
610 1.1 and 1.7 g N/kg in reactors A and B, respectively. Subsequently, NH₃ fluctuated with
611 an overall decreasing trend along the pH modification in both reactors, showing peaks
612 higher than 1.0 g N/kg mainly when pH was relatively high (i.e. ≥ 8.0) [Figure 3]. In
613 reactor A, NH₃ reached peaks of 1.4 (day 7) and 1.5 g N/kg (day 34), while NH₃ higher
614 than 1.5 g N/kg was repeatedly observed in reactor B (i.e. days 20, 27, 34 and 41).

615

616 In co-digestion. ~~The the~~ initial TAN ~~in co-digestion~~ was 3.0 g N/kg and slightly
617 increased to a maximum of 3.3, 3.6 and 3.3 g N/kg (day 61) in reactors A, B and C,
618 respectively [Figure 4d]. TAN subsequently decreased due to the reduced OFMSW
619 feeding and the progressive dilution used for HS-AD recovering, until a minimum of
620 1.9, 2.3 and 2.8 g N/kg was reached in reactors A, B and C, respectively (day 112). The
621 initial NH₃ was 2.0 g N/kg and progressively decreased in the three reactors alongside
622 pH. NH₃ peaked at 1.5 g N/kg (day 12) and 1.2-1.7 g N/kg (day 19), rapidly decreasing
623 to ≤ 0.1 g N/kg (day 23), similarly in all reactors. From this point, NH₃ was maintained
624 below 1.0 g N/kg in the three reactors. Thus, NH₃ was considerably reduced during co-
625 digestion alongside the reduction of OFMSW in the feed, since peaks higher than 1.0 g
626 N/kg were not observed from day 20 onwards, in contrast to mono-digestion reactors.

627

628 NH₃ inhibition was likely one of the main triggers of overloading in this study, since the
629 high NH₃ levels observed (i.e. ≥ 1.0 g N/kg) are normally associated ~~to~~ with
630 methanogenic inhibition and VFA accumulation in AD (Drosg, 2013; Rajagopal et al.,

631 | 2013). ~~(Drosg, 2013; Jokela & Rintala, 2003; Poggi-Varaldo et al., 1997)~~ Thus, despite
632 | each AD system might show particular NH₃ inhibition thresholds depending on the
633 | anaerobic consortia (Fricke et al., 2007; Westerholm et al., 2016), a gradual
634 | methanogenic adaptation to high levels of TAN (i.e. ≥ 4.0 g N/kg) might be crucial to
635 | increase OLR in semi-continuous HS-AD of OFMSW (Hartmann & Ahring, 2006;
636 | Rajagopal et al., 2013).

637

638 | In this study, a tradeoff was needed between the ‘undesired’ TAN buildup and the rapid
639 | TS removal observed, to reach HS-AD conditions (i.e. TS ≥ 10 %) with mono-digestion
640 | of OFMSW. For example, the different TS and TAN dynamics can be appreciated in
641 | mono-digestion reactor A from day 30, when TS fluctuated while TAN steadily
642 | increased [Figure 2]. Potential ammonia contingency strategies in AD, as increasing the
643 | substrate dilution, reducing the OLR, and/or increasing the MRT (Kayhanian, 1999;
644 | Rajagopal et al., 2013), would have lengthened considerably the experimental time, or
645 | even prevented to achieve HS-AD conditions (i.e. TS ≥ 10 %) with mono-digestion of
646 | OFMSW.

647

648 | 4 CONCLUSIONS

649 | In this study, reducing the effluent compared to the influent mass (i.e. ~~1-38~~18 %)
650 | permitted to extend the ~~biomass retention time~~MRT in semi-continuous mono-digestion
651 | of OFMSW, and obtain a specific biogas production of 229 L/kg VS added, due to the
652 | high biodegradability of OFMSW. However, the sole implementation of
653 | influent/effluent uncoupling was not sufficient to avoid reactor overload and
654 | acidification/acidification when reaching HS-AD conditions (i.e. TS ≥ 10 %). The

655 average OLR was 4.5 g VS/kg·d, whereas a maximum 11.5 % TS was reached. In
656 contrast, the addition of beech sawdust to OFMSW allowed to operate co-digestion
657 reactors with an average OLR of 8.3 g VS/kg·d, and reach a maximum 29.0 % TS. Co-
658 digestion lowered by 22 % the TAN content, though an average 186 L/kg VS added of
659 biogas was obtained. Overloading was related to the substrate biodegradability and
660 inhibitory content, since the maximum TAN content was 4.8 and 3.6 g N/kg, while the
661 biogas production was 166-192 and 59-71 L/kg VS Fed, for mono-digestion of
662 OFMSW and co-digestion of OFMSW and sawdust, respectively. Therefore, the
663 addition of sawdust, as an example of lignocellulosic substrate, to OFMSW (i.e. 1-2 g
664 VS-Sawdust/g VS-OFMSW) is an adequate strategy to stabilize HS-AD at very high TS
665 contents (i.e. 20-30 %). Nonetheless, a compromise must be found between increasing
666 the TS content and reducing the specific biogas production by co-digestion, since both
667 aspects strongly determine the HS-AD economy for OFMSW treatment.-

668

669

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822 TABLE AND FIGURE CAPTIONS

823 ~~Table 1: Summary of weekly operating parameters used in the semi-continuous reactors~~
824 ~~until last feeding was implemented (i.e. week 12)~~

825 **Table 21**: Bio-physical-chemical characterization of substrates.

826 **Table 32**: Physical-chemical characterization of inoculums.

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828 **Figure 1**: Experimental setup. 1) Reactor body; 2) reactor head; 3) feeding port; 4) gas
829 output; 5) gas measuring port; and 6) opening valves.

830 **Figure 2**: Mono-digestion of OFMSW: a) Organic loading rate; b) mass retention time;
831 c) total solids; d) total and free ammonia nitrogen (NH₃); e) cumulative biogas
832 production; and f) methane content. Black arrows represent the NaHCO₃ addition in

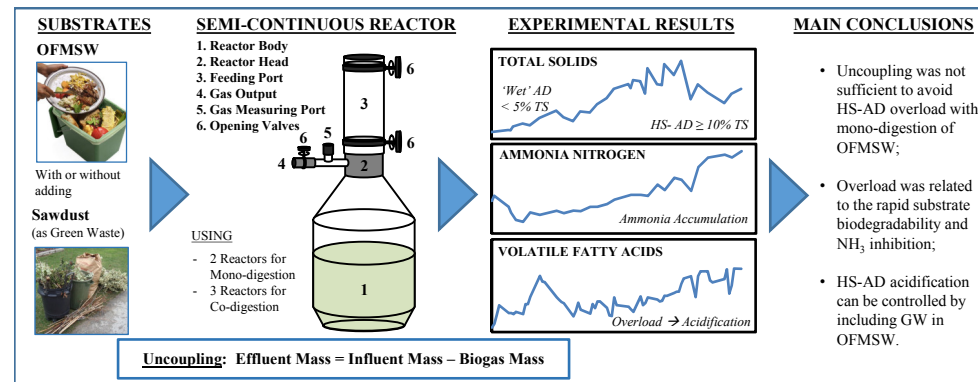
833 reactor A, while dotted arrows represent the FeCl₂ or inoculum addition in reactor B.

834 **Figure 3**: Mono-digestion of OFMSW: Volatile fatty acids and pH in a) reactor A; and
835 b) reactor B. Black arrows represent the NaHCO₃ addition in reactor A, while dotted
836 arrows represent the FeCl₂ or inoculum addition in reactor B.

837 **Figure 4**: Co-digestion of OFMSW and sawdust: a) Organic loading rate – parentheses
838 indicate the sole addition of OFMSW; b) mass retention time; c) total solids; d) total
839 and free ammonia nitrogen (NH₃); e) cumulative biogas production; and f) methane
840 content.

841 **Figure 5**: Co-digestion of OFMSW and sawdust: Volatile fatty acids and pH for a)
842 reactor A; b) reactor B; and c) reactor C.

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Highlights

- A reduced effluent compared to the influent extended the MRT in HS-AD of OFMSW.
- Uncoupling was not sufficient to avoid overload/acidification in HS-AD of OFMSW.
- Substrate overload in HS-AD of OFMSW was exacerbated by NH_3 inhibition.
- HS-AD overload can be controlled by adding green waste to OFMSW.

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1 **Semi-continuous Mono-digestion of OFMSW and Co-digestion of**
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4 **OFMSW with Beech Sawdust: Assessment of the Maximum**
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7 **Operational Total Solid Content**

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1 17 **ABSTRACT**

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3 18 In this study, mono-digestion of the organic fraction of municipal solid waste
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6 19 (OFMSW) and co-digestion of OFMSW with beech sawdust, simulating green waste,
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8 20 were used to investigate the maximum operational total solid (TS) content in semi-
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11 21 continuous high-solids anaerobic digestion (HS-AD). To alleviate substrate overloading
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13 22 in HS-AD, the effluent mass was relatively reduced compared to the influent mass,
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15 23 extending the mass retention time. To this aim, the reactor mass was daily evaluated,
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18 24 permitting to assess the reactor content removal by biogas production. During mono-
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20 25 digestion of OFMSW, the NH₃ inhibition and the rapid TS removal prevented to
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22 26 maintain HS-AD conditions (i.e. TS \geq 10 %), without exacerbating the risk of reactor
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25 27 acidification. In contrast, the inclusion of sawdust in OFMSW permitted to operate HS-
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27 28 AD up to 30 % TS, before acidification occurred. Therefore, including a lignocellulosic
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29 29 substrate in OFMSW can prevent acidification and stabilize HS-AD at very high TS
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31 30 contents (i.e. 20-30 %).
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37 32 **Keywords:** High-Solids Anaerobic Digestion; Influent/Effluent Uncoupling; Substrate
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40 33 Overloading; Acidification; Ammonia Inhibition.
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1 35 **1 INTRODUCTION**

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3 36 Anaerobic digestion (AD) of the organic fraction of municipal solid waste (OFMSW),
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6 37 including food waste (FW) and green waste (GW), is a particularly suited treatment
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8 38 biotechnology for energy and by-product recovery (Clarke, 2018; Mata-Álvarez, 2003).
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10 39 In AD, an organic waste is degraded to biogas, mainly composed by CH₄ and CO₂, and
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12 40 a partially stabilized organic digestate, by consortia of different microorganisms
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14 41 working in absence of oxidative species (i.e. O₂ and NO₃⁻) (Astals et al., 2015; Gerardi,
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16 42 2003).
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23 44 The sequential steps in AD include hydrolysis, acidogenesis, acetogenesis and
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25 45 methanogenesis, during which different inhibitory substances can be formed leading to
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27 46 inhibitory effects for the anaerobic microorganisms and/or even a complete AD failure.
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29 47 Depending on the concentration, free ammonia (NH₃), hydrogen sulfide (H₂S) and free
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31 48 ions (i.e. Na⁺) are some of the inhibitory substances in AD, affecting predominantly the
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33 49 methanogenic stage, either acetoclastic and/or hydrogenotrophic, and potentially
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35 50 resulting in the buildup of volatile fatty acids (VFA) and H₂ in the system (Astals et al.,
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37 51 2015; Chen et al., 2008). Meanwhile, the acetoclastic activity results into inorganic
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39 52 carbon (i.e. HCO₃⁻) release in AD, as an important source of pH buffering, minimizing
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41 53 the risk of reactor acidification (i.e. pH ≤ 6.0) by VFA accumulation (Gerardi, 2003).
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50 55 The interrelationship between the organic waste characteristics, operational conditions
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52 56 and reactor design determines the AD potential (Karthikeyan & Visvanathan, 2013;
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54 57 Mata-Álvarez, 2003). AD can be differentiated depending on the operational total solid
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56 58 (TS) content into 'wet' (i.e. TS < 10 %) and high-solids AD (HS-AD, i.e. TS ≥ 10 %)
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1 59 (Benbelkacem et al., 2015). HS-AD allows the use of a smaller reactor, reducing the
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3 60 need for water addition and minimizing the digestate production (Karthikeyan &
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5 61 Visvanathan, 2013; Pastor-Poquet et al., 2018). However, HS-AD drawbacks include
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7 62 the pervasive chances of reactor acidification due to substrate overload (Benbelkacem et
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9 63 al., 2015). Overloading is the consequence of the slow-growing methanogens being
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11 64 unable to cope with the rapid VFA and/or H₂ buildup resulting from
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13 65 acidogenesis/acetogenesis in HS-AD (Pavan et al., 2000). Furthermore, overloading is
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15 66 in many cases related to the presence of methanogenic inhibitors (Drosg, 2013), such as
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17 67 NH₃, due to the high protein content of OFMSW (Kayhanian, 1999).
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25 69 HS-AD of OFMSW is a mature technology, with most of the recently-constructed
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27 70 industrial plants targeting the semi-continuous HS-AD process (Mattheeuws, 2016).
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29 71 The focus of semi-continuous HS-AD lies on the maximization of the organic loading
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31 72 rate (OLR) that optimizes the methane yield and ensures an adequate organic removal at
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33 73 high TS contents (Benbelkacem et al., 2015; Hartmann & Ahring, 2006). In this line,
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35 74 depending on the organic waste used in HS-AD, the operational TS content is
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37 75 substantially lower than the feed TS, as the organic substrate is converted to biogas by
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39 76 methanogenesis (Pastor-Poquet et al., 2018).
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47 78 Therefore, HS-AD lies on a balance between maximizing the OLR and TS content,
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49 79 while minimizing the chances of reactor failure. Particularly, in order to startup HS-AD,
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51 80 the OLR needs to be increased relatively slowly, permitting the methanogens to grow
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53 81 and adapt to the new conditions. The transient (non-steady) OLR modification in HS-
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55 82 AD aims to find an optimum stationary (steady-state) operation to be used, avoiding
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1 83 acidification and maximizing the economy of the process (Angelidaki et al., 2006;
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3 84 Bolzonella et al., 2003). However, the risk of inhibition and failure is undesirably high
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5 85 under HS-AD startup, potentially requiring the implementation of recovering strategies
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8 86 (i.e. reactor content dilution) to minimize the influence of inhibitory substances, or even
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10 87 restarting the process when a significant methanogenic imbalance occurs (Fricke et al.,
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13 88 2007; Kayhanian, 1999).

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18 90 This study evaluated the highest tolerable TS content in semi-continuous HS-AD of
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20 91 OFMSW, by gradually increasing the OLR in semi-continuous reactors operated at
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22 92 55°C, until process failure occurred by acidification. Two feeding strategies were used:
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25 93 mono-digestion of OFMSW and co-digestion of OFMSW and beech sawdust – as a
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27 94 model lignocellulosic substrate, simulating the inclusion of GW in OFMSW. Aiming to
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29 95 minimize the risk of substrate overload, the mass retention time (MRT) was relatively
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31 96 extended by reducing the effluent compared to the influent mass, according to the daily
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33 97 mass content removal by biogas production observed in the semi-continuous reactors.
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40 100 **2 MATERIALS AND METHODS**

41 101 **2.1 Substrates and Inoculum**

42
43 102 The substrates used in this study were OFMSW and beech sawdust. OFMSW consisted
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45 103 of a mixture of household waste collected in Cassino (Italy), restaurant waste, spent
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47 104 coffee, and garden waste collected at the university facilities, with an approximated wet
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50 105 weight proportion of 45, 35, 15 and 5 % (w/w), respectively. OFMSW was minced
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53 106 twice to a particle size $\leq 5-10$ mm by an industrial mincer [REBER 9500NC, Italy],
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1 107 fully homogenized manually and stored in 5 L buckets at -20°C. During mincing and
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3 108 homogenization, no extra water was added to the raw substrate. A single 5 L bucket of
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5 109 OFMSW was thawed at room temperature overnight, as required to feed the semi-
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8 110 continuous reactors. Goldspan[®] beech sawdust with 1.0-2.8 mm particle size was used
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10 111 as co-substrate, to simulate biodegradable green/lignocellulosic waste.
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15 113 The inoculum for semi-continuous experiments was obtained from a pre-adapted ‘wet’
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17 114 AD (i.e. $TS \leq 5\%$) source reactor operated at 55°C. The pre-adaptation of 20 L sludge,
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19 115 collected from a mesophilic (35°C) digester treating buffalo manure and mozzarella
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21 116 whey (Capaccio, Italy), consisted of a 4-month progressive feeding of tap-water-diluted
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23 117 OFMSW at 55°C, in order to adapt the inoculum to the new substrate and temperature.
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29 119 Prior to start the mono-digestion experiments, the source reactor was kept unfed for 1
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31 120 month to consume/reduce the organic content, while continuing with the inoculum
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33 121 adaptation to the new substrate. Subsequently, the feeding with diluted OFMSW was
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35 122 resumed to recover methanogenesis. After 7 and 15 days from the feeding restart, 4 kg
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37 123 of sludge were taken from the source reactor, filtered through a 1 mm mesh and used to
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39 124 inoculate the mono-digestion reactors “A” and “B”, respectively. Therefore, the
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41 125 inoculum was slightly different in reactors A and B, as shown in section 3.1.
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49 127 During the mono-digestion experiments, the source reactor was periodically fed with
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51 128 diluted OFMSW and the mono-digestion reactors effluents, to maintain the reactor
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53 129 volume and methanogenic activity. Once the mono-digestion experiments ended, the
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55 130 source reactor was kept unfed for 1 month to serve as inoculum for the co-digestion
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131 experiments. Thus, 3.4 kg of reactor content were filtered through a 1 mm mesh and
132 used to inoculate each co-digestion reactor “A”, “B” and “C”.

133

134 **2.2 Experimental Setup**

135 The laboratory-scale semi-continuous reactors consisted of 5 L polyethylene
136 terephthalate (PET) bottles with a modified head allowing the (semi-)solid waste input,
137 reactor content withdrawal and biogas measurement [Figure 1]. The reactor port was a
138 polyvinyl chloride (PVC) flexible hosepipe with two valves, easing the reactor
139 loading/unloading while avoiding air intrusion. The biogas output, containing a
140 sampling septum, was connected to 5 L Tedlar® bags [Sigma-Aldrich, USA]. All
141 reactors were maintained at 55°C within a temperature-controlled TCF 400 oven
142 [ARGOLAB, Italy].

143

144 **2.3 Operation Strategy**

145 Two semi-continuous reactors for mono-digestion of OFMSW or three reactors for co-
146 digestion of OFMSW and sawdust were operated simultaneously in a drag-and-fill
147 mode. The semi-continuous reactors (i.e. kg) and the reactor influents/effluents (i.e. g)
148 were weighed on a ± 0.01 precision scale. The OLR was evaluated as the daily substrate
149 addition in terms of volatile solids (VS) divided by the reactor mass content (i.e. g
150 VS/kg·d), while the MRT was evaluated as the quotient between the reactor mass and
151 the daily effluent mass (i.e. days). Since the reactors were fed a maximum of 5 days per
152 week, 7-days moving average OLR and MRT were estimated. Moving-average
153 operational variables are well suited indicators of the immediately preceding operations
154 (i.e. feeding, dilution, reactor content removal) to discern about the risk of VFA buildup

1 155 in semi-continuous digesters. Moreover, expressing the operational conditions as a
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3 156 moving-average eases the comparison of digesters, when feeding days are not the same
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6 157 or an important mass removal occurs.
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8 158
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10 159 During each drag-and-fill operation, the reactor content was 1) homogenized before
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12 160 opening the system, 2) sampled and 3) analyzed mainly for pH and alkalinity – since pH
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14 161 had to be maintained over 6.5, as an important methanogenic inhibition might take place
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16 162 below this threshold (De Vrieze et al., 2012; Gerardi, 2003). Depending on the pH and
17
18 163 alkalinity, 4) the proper amount of substrate was used or diluted as needed, 5) prior to
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20 164 be fed to the reactors. Finally, 6) the reactor content was homogenized once again,
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22 165 while the Tedlar® bags were checked for biogas production and subsequently emptied.
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30 167 To increase the reactor TS content from ‘wet’ AD (i.e. TS < 5 %) to HS-AD (i.e. TS ≥
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32 168 10 %), the OLR was controlled by increasing/decreasing the daily amount of substrate
33
34 169 and/or tap water addition based on the methanogenic activity, and aiming to minimize
35
36 170 the substrate overload. To evaluate the differences in the reactor performance, mono-
37
38 171 digestion reactors were fed in parallel using different OLR/MRT in each reactor, as
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40 172 shown in section 3.2. Subsequently, co-digestion reactors were also operated in parallel
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42 173 at three different OLR/MRT. In each reactor, the methanogenic activity was roughly
43
44 174 associated with the relative increase of the pH and inorganic carbon alkalinity (ALK_P),
45
46 175 the reduction of the reactor mass content and the biogas production compared to
47
48 176 previous operational values, as also mentioned in section 3.2. For example, a relative
49
50 177 pH and ALK_P increase of approximately 0.5 pH units and 0.3 g CaCO₃/kg, respectively,
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52 178 alongside a reactor mass removal of about 30-50 g/d and a specific biogas production
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1 179 higher than 250 mL/kg reactor content·d were associated with ongoing methanogenesis,
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3 180 indicating that the OLR could be maintained or relatively increased. Similarly, the
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5 181 relative increase of intermediate alkalinity (ALK_I) (i.e. 0.5 g Acetic Acid/kg) was used
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8 182 as a preliminary indicator of the potential VFA buildup and risk of substrate overload
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11 183 (Lahav et al., 2002).

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15 185 All these parameters were further complemented with the user's evaluation of the
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18 186 previous operation, in order to decide for the daily feed/dilution to be used. Thus, all
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21 187 reactors were started with a low OLR (i.e. 2 g VS/kg·d) that was gradually increased to
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23 188 increase the TS content. As reactor performance deteriorated with increasing OLR, the
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25 189 reactor feeding was reduced/stopped to prevent acidification (i.e. pH ≤ 6.0).

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30 191 The reactor mass was maintained constant by reducing the effluent compared to the
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32 192 influent mass, according to the observed reactor mass content removed by biogas
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35 193 production from the previous operation. With this strategy, the MRT was relatively
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37 194 extended, aiming to promote the methanogenic adaptation in case of overloading. Semi-
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40 195 continuous reactors were fed until acidification occurred. From this point, feeding was
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42 196 stopped and reactor dilution and/or inorganic salt addition (i.e. NaHCO₃ and FeCl₂)
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45 197 were tested as recovering strategies. A summary of the weekly operational variables is
46
47 198 presented as Supplementary Information.

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51 52 200 **2.4 Bio-Physical-Chemical Analyses**

53
54 201 The pH, ALK_P and ALK_I were determined from the supernatant of solid and semi-solid
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57 202 samples (Lahav et al., 2002), after diluting the sample with distilled water,
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1 203 homogenization and centrifugation at 6000 rpm for 15 min (EPA, 2015). The TS and
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3 204 VS content, total Kjeldahl (TKN) and ammonia (TAN) nitrogen, and the total H₂S were
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5 205 determined by the standard methods (APHA, 1999). The NH₃ was approximated as a
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7 206 function of TAN and pH (Astals et al., 2015). The VFA (acetic, propionic, butyric and
8
9 207 valeric acids) were measured with an LC-20AD HPLC [Shimadzu, Japan], mounting a
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11 208 Rezex ROA-Organic Acids 8+ column coupled to a 210 nm UV detector, and using
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13 209 0.0065 M H₂SO₄ at 0.6 mL/min as mobile phase. The biogas composition (CH₄, CO₂
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15 210 and H₂) was analyzed with a 3400 GC-TCD [Varian, USA], using argon as carrier gas.
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17 211
18
19 212 The biomethane potential (BMP) test for OFMSW used 3.0 g of substrate, 50.0 g of
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21 213 source inoculum, 40.0 g of distilled water and 0.10 g of NaHCO₃ in 280 mL bottles (6
22
23 214 replicates), with an inoculum-to-substrate ratio (ISR) of 2.0 g VS/g VS. The BMP test
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25 215 for sawdust used 1.0 g substrate and 50.0 g of inoculum in 160 mL bottles (3 replicates),
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27 216 with an ISR of 1.0 g VS/g VS. BMP tests were performed according to Angelidaki and
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29 217 Sanders (2004) and Holliger et al. (2016). In the BMP test for OFMSW, the distilled
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31 218 water and NaHCO₃ addition served to minimize the chances of inhibition (i.e. by NH₃)
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33 219 and acidification, respectively. In contrast, NH₃ build-up and acidification were not
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35 220 expected in the BMP test of sawdust, due to the low nitrogen content and the reduced
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37 221 biodegradability of sawdust, as thoroughly discussed in next section, permitting also to
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39 222 use a lower ISR. Both BMP tests lasted longer than 100 days. Blank assays included the
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41 223 inoculum and further distilled water compensating for the absence of substrate, using
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43 224 three replicates in each BMP. Inoculum activity assays using a reference substrate were
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45 225 not performed.
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1 227 The BMP was the normalized methane production ($P = 1$ bar, $T = 0^{\circ}\text{C}$), excluding the
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3 228 methane production of the inoculum, per unit of substrate VS added. The gas production
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5 229 was evaluated with a two-vessel displacement system, with the first vessel containing 4
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8 230 N NaOH to capture CO_2 and the second vessel containing water to be ‘displaced’. The
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10 231 bottles were sealed with butyl rubber stoppers and aluminum crimps and flushed with
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12 232 helium, before adding 0.2 mL of 10 g/L Na_2S piercing the septum to ensure an adequate
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14 233 redox potential (Angelidaki & Sanders, 2004). All bottles were incubated at 55°C and
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16 234 agitated only while measuring the gas production.
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22 236 **2.5 Statistical Analyses**

25 237 The Dixon’s test for BMP outliers was applied as recommended by Holliger et al.
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27 238 (2016). The unpaired t-test of Microsoft Excel 2016 (Microsoft, USA) was applied to
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29 239 determine the statistical significance of experimental data, using the two-tail p-value at
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31 240 95 % confidence.
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37 243 **3 RESULTS AND DISCUSSION**

38 244 **3.1 Bio-Physical-Chemical Characterization of Substrates and Inoculum**

39 245 OFMSW showed a TS of 26 %, a VS/TS ratio of 0.93 and a TKN of 24.8 g N/kg TS, in
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41 246 agreement with real source-sorted OFMSW (Angelidaki et al., 2006; Bolzonella et al.,
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43 247 2006; Jokela & Rintala, 2003). The high VS/TS ratio of OFMSW (i.e. > 0.9) indicated
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45 248 minimal presence of inert materials (Pavan et al., 2000). Sawdust showed a TS of 94 %
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47 249 and a VS/TS ratio of 0.99, similar to those obtained by Brown and Li (2013) for 40°C -
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49 250 dried yard waste, suggesting that beech sawdust could simulate GW. The BMP of
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1 251 OFMSW and sawdust was 497 and 161 NmL CH₄/g VS, respectively. Table 1 shows
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3 252 the bio-physical-chemical characterization of OFMSW and sawdust. Despite the
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5 253 thorough mincing and homogenization, minor modifications were observed in the
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8 254 OFMSW characterization (i.e. TS, TKN or BMP), mainly attributed to the substrate
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10 255 heterogeneity.
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15 257 The inoculum in mono-digestion reactors A and B showed a common TS and TKN of
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17 258 2.8 % and 161 g N/kg TS, respectively. An initial acetic acid concentration of 2.30 and
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19 259 3.30 g/kg was observed in reactors A and B, respectively, being this difference
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21 260 associated with the later inoculation of reactor B than reactor A. The inoculum used in
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23 261 co-digestion reactors showed a TS of 2.5 %, a TKN of 139 g N/kg TS and an acetic acid
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25 262 concentration of 0.02 g/kg. The inoculum compositions are shown in Table 2.
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32 264 **3.2 Semi-continuous Operation – Increasing the TS Content**

33 265 **3.2.1 Mono-digestion of OFMSW**

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35 266 Mono-digestion results are summarized in Figures 2 and 3. The weekly-averaged results
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37 267 were also included as Supplementary Information. The 7-days average OLR in reactors
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39 268 A and B was varied from an initial 2.4 (day 6) and 6.0 (day 13) g VS/kg·d to 4.9 and 5.5
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41 269 g VS/kg·d, respectively, on day 17 [Figure 2a]. Thus, a common OLR (i.e. around 5 g
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43 270 VS/kg·d) was achieved, aiming to compensate for the 1-week-lagged inoculation in
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45 271 reactor B. After two days with no feed, feeding was resumed in reactor B on day 20, but
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47 272 not in reactor A due to the low pH (i.e. 6.4) [Figure 3a]. As pH recovered in reactor A
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49 273 (i.e. from 6.4 on day 21, to 7.6 on day 29) due to methanogenesis activity, feeding was
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51 274 resumed. During the same period, ALK_P in reactor A increased alongside pH from 1.1
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1 275 to 2.7 g CaCO₃/kg (data not shown), as an indicator of ongoing methanogenesis. By day
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3 276 45, a maximum OLR of 6.8 and 8.5 g VS/kg·d was reached in reactors A and B,
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5
6 277 respectively. After day 48, the OLR required progressive reduction to minimize the risk
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8 278 of acidification. The last feeding in reactors A and B was implemented on days 78 and
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10 279 73, respectively, as both reactors showed pH ≤ 6.5 and CH₄ content ≤ 40 % [Figure 2f].
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13 280 From this point, mono-digestion reactors were left unfed aiming to promote the
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15 281 recovery of methanogenesis.
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20 283 The OLR in reactor B was averagely about 1.5 g VS/kg·d higher than that used in
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22 284 reactor A during the whole experiment (p < 0.001), explaining the relatively faster
23
24 285 acidification observed in reactor B. Thus, prior to the occurrence of acidification,
25
26 286 reactor B was fed with an average 35 g VS/d, significantly higher than the 26 g VS/d
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28 287 used for reactor A (p = 0.03). The initial MRT was 55 (day 6) and 29 days (day 13) for
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30 288 reactors A and B, respectively, and was gradually increased to maintain the
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33 289 methanogenic performance at higher OLR [Figure 2b]. Noteworthy, the MRT and OLR
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35 290 in these semi-continuous reactors did not show an inverse pattern, since the dilution as
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37 291 well as the influent and effluent mass flows used were different to account for the
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39 292 organic removal.
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47 294 Uncoupling the influent and effluent mass flows in the semi-continuous reactors, based
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49 295 on the HS-AD reactor content removal by methanogenesis, permitted to increase the
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51 296 MRT and OLR simultaneously. In this study, the MRT was considered as a more suited
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53 297 indicator in HS-AD than the hydraulic retention time (HRT), since both the specific
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55 298 weight of the influent/effluent and the reactor mass content varied, in contrast to ‘wet’
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1 299 AD (Pastor-Poquet et al., 2018). As an example, the occurrence of methanogenesis led
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3 300 to a 60 g removal of the reactor mass content in both mono-digestion reactors from day
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5 301 37 to 41 (data not shown). Prior to the occurrence of reactor acidification, the weekly
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7 302 effluent mass was significantly higher than the influent (i.e. 18 %; $p = 0.03$) to maintain
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9 303 the mono-digestion reactors mass content constant.
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12
13 305 The MRT-uncoupling concept was proposed by Richards et al. (1991) and was used by
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15 306 Kayhanian and Rich (1995) to operate a pilot-scale semi-continuous HS-AD reactor fed
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17 307 with OFMSW. In this study, uncoupling the influent and effluent in HS-AD promoted
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19 308 the methanogenic adaptation to overloading conditions and/or the buildup of inhibitors
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21 309 (i.e. NH_3) during the OFMSW degradation. Noteworthy, the MRT must be longer than
22
23 310 the doubling time of methanogens (i.e. 20-30 days) to avoid their ‘washout’ from
24
25 311 continuous HS-AD reactors, while the methanogenic doubling time might lengthen
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27 312 considerably in presence of inhibitory substances (i.e. NH_3) (Drosg, 2013; Gerardi,
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29 313 2003; Rittman & McCarty, 2001). Therefore, extending the MRT resulted in a more
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31 314 stable HS-AD operation (Hartmann & Ahring, 2006; Rajagopal et al., 2013), though the
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33 315 sole implementation of influent-effluent uncoupling was not sufficient to avoid HS-AD
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35 316 overloading and acidification during mono-digestion of OFMSW.
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47 318 **3.2.2 Co-digestion of OFMSW and Sawdust**

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49 319 Co-digestion results are summarized in Figures 4 and 5. The 7-days average OLR was
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51 320 increased from 4.5-4.9 g VS/kg·d (day 6) up to 10.9, 12.1 and 12.6 g VS/kg·d (day 23)
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53 321 in reactors A, B and C, respectively. To avoid acidification, feeding was stopped in
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55 322 reactors A and B from day 26, while the OLR was only reduced to 5.0 g VS/kg·d in
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1 323 reactor C [Figure 4a]. As pH recovered (i.e. ≥ 7.0) [Figure 5], feeding was resumed in
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3 324 reactors A and B. A maximum OLR of 14.8 g VS/kg·d was reached in reactor C (day
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5 325 47) using a sawdust/OFMSW ratio of 2.1 g VS/g VS, prior to the occurrence of reactor
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8 326 acidification (day 56). The maximum OLR in reactor B was 15.1 g VS/kg·d (day 55)
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10 327 using a sawdust/OFMSW ratio of 1.6 g VS/g VS, while an OLR of 16.0 g VS/kg·d was
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12 328 reached in reactor A during the same period, using a sawdust/OFMSW ratio of 1.3 g
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14 329 VS/g VS. The last feeding in reactors A and B was performed on day 76, as a slight but
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16 330 continued drop in pH [Figure 5] and CH₄ [Figure 4f] was observed in both reactors.
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23 331
24 332 The average OLR used for co-digestion was two times higher than that for mono-
25 333 digestion (i.e. 8.3 vs. 4.5 g VS/kg·d, respectively; $p < 0.001$), due to the lower
26 334 biodegradability of sawdust, though the OLR only due to OFMSW was similar in both
27 335 cases (i.e. 4.1 vs. 4.5 g VS/kg·d, respectively; $p = 0.07$). Thus, a maximum OLR of 7.5-
28 336 8.0 g VS/kg·d related to the sole supplementation of OFMSW was used in the three co-
29 337 digestion reactors on day 21, while the OLR solely due to OFMSW was subsequently
30 338 maintained below 6.0 g VS/kg·d, as sawdust was increased in the feeding mixture
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33 339 [Figure 4a]. In terms of average VS fed, reactor C was operated under relatively more
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35 340 stressing feeding conditions than reactors A and B (i.e. 53 vs. 44 g VS/d, respectively; p
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37 341 = 0.15), being again the fastest occurrence of reactor acidification related to the highest
38 342 VS fed.
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51 343
52 344 The initial MRT was higher than 168 days (day 6) and was decreased to 30 days (day
53 345 17), similarly in the three reactors [Figure 4c]. From this point, the MRT reached an
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55 346 average of 85 days (day 35) and was subsequently reduced to an average of 37 days
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1 347 (day 53) in all reactors, before being progressively increased to minimize the substrate
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3 348 overload. The MRT was significantly lower in co-digestion than mono-digestion (i.e. 69
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5 349 vs. 92 days, respectively; $p < 0.001$), as lower MRT were predominantly linked to the
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8 350 higher OLR used in co-digestion.
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11 352 **3.3 Influence of the Substrate Composition on the TS Increase**

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13 353 The OLR/MRT control in the mono-digestion reactors fed with OFMSW permitted to
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15 354 increase the TS content, balancing the VFA accumulation with the rapid organic
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18 355 degradation observed [Figures 2 and 3]. Reactors A and B were started at TS = 2.8 %
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20 356 and reached a maximum of 10.7 (day 79) and 11.7 % (day 69), respectively [Figure 2c],
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22 357 being these TS slightly higher than the lower HS-AD threshold (i.e. TS ≥ 10 %). The
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24 358 highest TS in the semi-continuous reactors did not coincide with the maximum OLR,
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26 359 but were predominantly associated with low pH (i.e. ≤ 6.5), when methanogenesis was
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28 360 potentially inhibited. In this line, a gradual increase of the VS/TS ratio (data not shown)
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30 361 was observed in both reactors from 0.69 (day 0) to 0.82 (day 40), reaching a maximum
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32 362 value of 0.87, prior acidification occurred on days 79 and 76 in reactors A and B,
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34 363 respectively.
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45 365 The highest TS and VS/TS observed in semi-continuous HS-AD of OFMSW were
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47 366 associated with acidification and indicate a reduced VS degradation alongside inhibitory
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49 367 conditions. Particularly, the lowest HS-AD threshold (i.e. TS = 10 %) using OFMSW
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51 368 was reached only under extreme overloading. A more stable HS-AD fed with an easily
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53 369 biodegradable OFMSW (i.e. FW) is also associated with a TS increase alongside
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57 370 overloading/inhibitory conditions. For example, Tampio et al. (2014) reported a TS
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1 371 increase from 7 to 8 % during 400 days of semi-continuous AD fed with FW, though TS
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3 372 rapidly reached 11 % during the next 50 days of operation, when reactor inhibition was
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6 373 likely occurring. In the same line, Bolzonella et al. (2003) reported a TS increase from 5
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8 374 to 15 % during the initial 60 days of continuous AD pilot-scale startup fed with
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10 375 OFMSW, being the maximum TS associated with the highest total VFA observed (i.e.
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12 376 2.8 g Acetic Acid/L). All these results were likely related to methanogenesis inhibition,
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14 377 since the VFA accumulation affects the hydrolysis/acidogenesis rates, hampering the
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16 378 organic removal in HS-AD (Vavilin et al., 2008).
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23 380 The maximum TS obtained in this study for semi-continuous HS-AD of OFMSW
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25 381 should be considered as indicative (only) of those obtainable in steady-state digesters,
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27 382 since the transient/acidification conditions potentially reduced the VS removal. Thus,
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29 383 the operational TS content of stable digesters fed with the easily biodegradable content
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31 384 of OFMSW (i.e. FW) might be lower than those observed along non-steady-state
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33 385 conditions. This is a further indication that a steady-state semi-continuous reactor using
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35 386 an easily biodegradable OFMSW as a substrate might not be operated within the HS-
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37 387 AD threshold (i.e. $TS \geq 10\%$).
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45 389 Co-digestion permitted to increase TS from 2.5 % (day 0) up to a maximum of 33.2
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47 390 (day 79), 26.7 (day 76) and 27.0 % (day 57) in reactors A, B and C, respectively [Figure
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49 391 4c]. Hence, the maximum TS reached in co-digestion before the reactors acidified (i.e.
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51 392 $29.0 \pm 2.8\%$) was considerably higher than the lower HS-AD threshold (i.e. $TS \geq 10\%$)
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53 393 and the maximum TS of mono-digestion (i.e. $11.5 \pm 0.5\%$), due to the addition of
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55 394 sawdust to OFMSW. The highest TS was related again to acidified (i.e. $pH < 6.5$) or
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1 395 acidifying (i.e. downward trend on pH/CH₄ content) conditions, as observed for mono-
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3 396 digestion. Thus, the VS/TS ratio in co-digestion (data not shown) increased from 0.65
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5 397 (day 0) to 0.90 (day 40), reaching a maximum of 0.95 before reactors acidified (day 76),
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8 398 due to both the higher VS/TS ratio of sawdust and the reduced VS removal during
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10 399 inhibitory conditions.

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15 401 These results showed that the particular characteristics of OFMSW determined the
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17 402 maximum operating TS content in semi-continuous HS-AD. Co-digestion of OFMSW
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19 403 and sawdust resulted in approximately three times higher TS than mono-digestion (i.e.
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21 404 29.0 and 11.5 %, respectively). The inclusion of sawdust in OFMSW favored the rapid
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23 405 TS and OLR increase compared to mono-digestion due to the higher TS and the lower
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25 406 biodegradability of sawdust, as demonstrated by the substantially lower BMP of
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27 407 sawdust than that of OFMSW (i.e. 161 and 497 NmL CH₄/g VS, respectively) [Table
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29 408 1]. Indeed, lignocellulosic materials (i.e. GW) are normally associated with a reduced
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31 409 biodegradation rate, compared to more easily degradable substrates (i.e. FW), due to the
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33 410 high lignin content hampering hydrolysis (Brown & Li, 2013; Mancini et al., 2018a;
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35 411 Vavilin et al., 2008), being also beneficial to limit the VFA buildup in HS-AD. On the
36
37 412 other hand, TAN was 22 % lower during co-digestion than mono-digestion (i.e. 2.9 vs.
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39 413 3.7 g N/kg, respectively; $p < 0.001$) [Figures 2 and 4], due to the lower TKN of sawdust
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41 414 [Table 1]. Noteworthy, the TAN accumulation was likely promoting methanogenic
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43 415 inhibition in this study, as further discussed in section 3.5. Therefore, using sawdust –
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45 416 as GW – was also adequate to adjust the carbon-to-nitrogen (C/N) ratio in HS-AD of
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47 417 OFMSW.

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3 420 **3.4 Main Indicators of Substrate Overload**4
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6 421 **3.4.1 Evolution of pH and VFA**

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8 422 pH in mono-digestion reactor A decreased from 8.1 to 6.4 due to the rapid acetic acid
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10 423 buildup (i.e. from 3.00 to 9.00 g/kg) observed during the initial 20 days of operation
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12 424 [Figure 3a]. As feeding was stopped from day 20 to 29, pH reached 7.6, while acetic
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14
15 425 acid decreased below 0.70 g/kg right afterwards (day 34). Propionic, butyric and valeric
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17 426 acids gradually increased from < 0.15 g/kg (day 0) to 5.00, 4.00 and 1.10 g/kg (day 79),
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19
20 427 respectively. From day 79, pH dropped from 7.1 to 6.1, linked to a sudden acetic acid
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22 428 increase from 3.00 to 5.00 g/kg, and the subsequent CH₄ content drop from 56 to 37 %
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25 429 [Figure 2f].

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30 431 Mono-digestion reactor B was relatively more stressed than reactor A, as indicated by
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32 432 the wider acetic acid fluctuations (i.e. ± 4.00 g/kg) and the rapid accumulation of
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35 433 propionic acid from 1.20 (day 7) to 5.80 g/kg (day 73) [Figure 3b]. The VFA fluctuation
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37 434 is in line with the fact that methanogens grow relatively slower than the
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40 435 hydrolytic/acidogenic microorganisms in AD (De Vrieze et al., 2012; Gerardi, 2003).
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42 436 Thus, the higher OLR used in reactor B led to a more pronounced
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45 437 methanogenic/acidogenic imbalance, exacerbating the VFA accumulation. The VFA
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47 438 buildup led to a pH decrease from 8.4 to 6.2 in reactor B during the whole experimental
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50 439 period, while a significant acetoclastic inhibition occurred from day 70 to 73, when
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52 440 acetic acid abruptly increased from 2.70 to 5.80 g/kg.

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1 442 pH in co-digestion reactor A gradually decreased from 8.7 to 6.4 along the experimental
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3 443 period, showing a minimum of 6.1 associated with a peak of acetic acid of 8.30 g/kg
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5 444 (day 26) [Figure 5a]. Acetic acid was considerably consumed (i.e. < 0.36 g/kg) by day
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8 445 47 due to ongoing methanogenesis, and progressively increased thereafter by
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10 446 overloading. Similarly, pH in reactor B showed a minimum of 6.3 when acetic acid
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12 447 peaked at 8.20 g/kg (day 26) [Figure 5b], while the acetic acid was extensively
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14 448 consumed (i.e. < 0.35 g/kg) by day 41 prior to increase again steadily. In reactor C,
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16 449 acetic acid had a similar evolution with a maximum of 7.20 g/kg (day 26) [Figure 5c],
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18 450 while pH dropped to 6.0 on day 57, associated with a sharp acetic acid build-up from
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20 451 1.00 to 3.70 g/kg. Propionic, butyric and valeric acids increased from 0.50, 0.14 and
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22 452 0.00 g/kg (day 0) to a maximum range of 3.00-3.50, 2.90-3.20 and 2.50-2.60 g/kg,
23
24 453 respectively, obtained right after acidification occurred on day 79 in reactors A and B,
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26 454 and on day 56 in reactor C. The pH was relatively lower (i.e. 2 %; $p = 0.13$) and total
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28 455 VFA was relatively higher (i.e. 5 %; $p = 0.25$) during mono-digestion than co-digestion,
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30 456 likely due to the faster degradation rates but also the higher release of inhibitory
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32 457 compounds related to OFMSW than sawdust, as discussed in section 3.3.
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36 459 Feeding the reactors a maximum of 5 days per week influenced the reactor dynamics,
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38 460 since pH increased and VFA – mainly acetic acid – decreased during the periods with
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40 461 no feed. The pH and VFA modifications [Figures 3 and 5] were associated with the TS
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42 462 removal, as mentioned before, and also affected the biogas production/composition, and
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44 463 the TAN buildup [Figures 2 and 4]. As an example, in co-digestion reactor A, pH
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46 464 increased from 7.0 to 7.9 from day 37 to 41, while acetic acid decreased from 4.30 to
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1 465 2.40 g/kg, triggering a biogas production of 0.6 L/kg reactor content and a methane
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3 466 content increase from 59 to 70 % [Figure 4 and 5].
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8 468 **3.4.2 Biogas Production and Composition**

10 469 Mono-digestion of OFMSW resulted in a cumulative biogas production of 65 and 66
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13 470 L/kg reactor content in reactor A and B, respectively [Figure 2e]. Biogas production
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15 471 was mainly correlated to the acetic acid consumption [Figure 3a], as mentioned in the
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18 472 previous subsection. For example, 21 L/kg reactor content of biogas were measured
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20 473 during the initial 20 days of reactor A, before acetic acid accumulated and biogas
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23 474 production slowed down.
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27 476 Biogas composition measurements started on day 60 showing an average of 63 % CH₄
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29 477 in both mono-digestion reactors [Figure 2f], which subsequently fluctuated showing a
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32 478 downward trend alongside the VFA accumulation. The CH₄ content dropped below 40
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35 479 % in both reactors right after biogas production definitely ceased on days 78-79. The
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37 480 reduction of CH₄ content in the biogas is also an indicator of AD imbalance, though it
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40 481 might be inappropriate to assess rapid changes in the reactor performance (Drosg,
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42 482 2013). The highest H₂ concentration (data not shown) was 1.8 and 1.1 % on day 59 in
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45 483 reactors A and B, respectively, while H₂ remained below 0.8 % in both reactors during
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47 484 the rest of the experiment. The presence of H₂ indicated that the hydrogenotrophic
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49 485 methanogens were unable to cope with the rapid H₂ production from acidogenesis, since
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52 486 H₂ higher than 1-2 % in the gas phase is normally associated with AD overloading
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55 487 (Drosg, 2013; Molina et al., 2009).
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1 489 Co-digestion in reactor A led to a cumulative biogas production of 48 L/kg reactor
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3 490 content, while 49 and 27 L/kg reactor content were observed in reactors B and C,
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5 491 respectively [Figure 4e]. In spite of the higher OLR used in co-digestion, the biogas
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7 492 production was considerably lower than that obtained with mono-digestion (i.e. 65 L/kg
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9 493 reactor content). Thus, the specific biogas production was 229 ± 20 L/kg VS added in
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11 494 mono-digestion and 86 ± 18 L/kg VS added in co-digestion (i.e. 62 % lower), due to the
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13 495 reduced biodegradability of sawdust.
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20 497 The CH₄ content [Figure 4f] reached a peak of 75 % during the first two weeks of
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22 498 operation in the three co-digestion reactors, but it decreased subsequently as VFA
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24 499 accumulated [Figure 5]. A minimum 43 % CH₄ was detected in reactor A associated
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26 500 with the last biogas production observed (day 82), while a sharp drop from 60 to 29 %
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28 501 CH₄ was observed in reactor C right after day 60. H₂ was detected at 0.3 % in the three
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30 502 co-digestion reactors on day 23 (data not shown). Thereafter, H₂ was not detected in
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32 503 reactor A, while reactor B showed a single H₂ peak of 1.5 % on day 70, right after the
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34 504 reactor was accidentally opened to the atmosphere. In reactor C, H₂ peaks of 1.7, 1.2
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36 505 and 1.6 % were observed on days 41, 47 and 58, respectively, supporting the occurrence
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38 506 of a more extensive overload in this reactor.
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48 508 **3.5 Testing Recovering Strategies**

49 509 Once acidification occurred, feeding was stopped and some recovering strategies were
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51 510 tested to resume methanogenesis. In mono-digestion reactor A, a 3 M NaHCO₃ buffer
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53 511 solution was added on days 83 and 84 to raise the pH (i.e. from 6.2 to 6.8) within a
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55 512 suitable range for methanogens (i.e. 6.5-7.0). Adding NaHCO₃ is normally used to
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1 513 counteract acidification when digesters show a reduced ALK_p (Chen et al., 2008;
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3 514 Holliger et al., 2016). However, methanogenesis did not recover after more than 20
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6 515 days.
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10 517 On day 76, $FeCl_2$ was supplemented to mono-digestion reactor B in a higher amount
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12 518 than the stoichiometric, to precipitate the total H_2S in the system (i.e. 30 mg H_2S/kg ,
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14 519 data not shown). However, $FeCl_2$ overdosing resulted in a pH drop from 6.3 to 5.7 (days
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16 520 76-77). Thus, 2 M $NaHCO_3$ solution was rapidly added to recover the pH to 6.6 (day
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18 521 77). Both Fe^{2+} and/or Fe^{3+} can be used to precipitate sulfide in AD, but Fe^{2+} was
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20 522 preferred in this study to avoid the inclusion of a strong electron acceptor (i.e. Fe^{3+}) that
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22 523 could react with organic compounds in the anaerobic digester (i.e. $Fe^{3+} + 1/2 H_2 \rightarrow Fe^{2+}$
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24 524 + H^+ , $\Delta G^{\circ} \ll 0$) (Fermoso et al., 2015; Rittman & McCarty, 2001). After 2 weeks of
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26 525 methanogenic inhibition (day 90), 200 g of ‘wet’ AD inoculum from the source reactor
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28 526 were added to reactor B, allowing a gradual methanogenic recovery, associated with an
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30 527 increase of pH from 6.9 to 7.3 [Figure 3b] and CH_4 content from 20 to 52 % [Figure 2f],
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32 528 until the end of the reactor operation.
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36 530 Aiming to recover methanogenesis in all co-digestion reactors, water was progressively
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38 531 added to dilute the effect of potential methanogenic inhibitor(s). The progressive
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40 532 addition of low amounts of water in co-digestion reactors permitted to maintain HS-AD
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42 533 conditions (i.e. $TS \geq 10\%$), thanks to the elevated TS content reached before reactors
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44 534 acidified (i.e. $TS \geq 30\%$). Dilution was performed in reactors A and B from day 79 and
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46 535 in reactor C from day 62. In reactor A and B an average of 180 and 170 mL of water
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1 536 was used, respectively, on days 79, 82, 84, 91 and 98, while an average of 160 mL of
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3 537 water was added to reactor C on days 62, 63, 68 and 91.
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8 539 In conclusion, neither water, nor buffer addition permitted to recover
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10 540 acidified/acidifying HS-AD reactors, probably because of the important imbalance
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12 541 between methanogens and acid-producers in the system (Gerardi, 2003). In these
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14 542 conditions, inoculum addition might be the only way to recover an acidified HS-AD
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16 543 reactor, though emptying and re-inoculating the reactor might be necessary (Fricke et
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18 544 al., 2007). Another strategy to prevent reactor acidification and/or enhance the digester
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20 545 performance is trace element (i.e. Se, Ni, Co, Mo, W) addition (Fermoso et al., 2015;
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22 546 Mancini et al., 2018b). However, this was out of the scope of this manuscript.
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30 548 **3.6 Ammonia Buildup**

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32 549 In this study, overloading was associated with the high OLR used, but also with the NH₃
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34 550 buildup, in the semi-continuous reactors. The high OLR and the degradation of the
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36 551 protein content of OFMSW increased the TAN content in both mono-digestion reactors
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38 552 [Figure 2d]. TAN ranged from 3.4 g N/kg (day 0) to a maximum of 4.8 and 4.9 g N/kg
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40 553 in reactors A and B (day 104), respectively, with both reactors showing a minimum
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42 554 TAN of 3.0 g N/kg around day 20. The initial NH₃ was 1.1 and 1.7 g N/kg in reactors A
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44 555 and B, respectively. Subsequently, NH₃ fluctuated with an overall decreasing trend
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46 556 along the pH modification in both reactors, showing peaks higher than 1.0 g N/kg
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48 557 mainly when pH was relatively high (i.e. ≥ 8.0) [Figure 3]. In reactor A, NH₃ reached
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50 558 peaks of 1.4 (day 7) and 1.5 g N/kg (day 34), while NH₃ higher than 1.5 g N/kg was
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52 559 repeatedly observed in reactor B (i.e. days 20, 27, 34 and 41).
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3 561 In co-digestion, the initial TAN was 3.0 g N/kg and slightly increased to a maximum of
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5 562 3.3, 3.6 and 3.3 g N/kg (day 61) in reactors A, B and C, respectively [Figure 4d]. TAN
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7 563 subsequently decreased due to the reduced OFMSW feeding and the progressive
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9 564 dilution used for HS-AD recovering, until a minimum of 1.9, 2.3 and 2.8 g N/kg was
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11 565 reached in reactors A, B and C, respectively (day 112). The initial NH₃ was 2.0 g N/kg
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13 566 and progressively decreased in the three reactors alongside pH. NH₃ peaked at 1.5 g
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15 567 N/kg (day 12) and 1.2-1.7 g N/kg (day 19), rapidly decreasing to ≤ 0.1 g N/kg (day 23),
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17 568 similarly in all reactors. From this point, NH₃ was maintained below 1.0 g N/kg in the
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19 569 three reactors. Thus, NH₃ was considerably reduced during co-digestion alongside the
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21 570 reduction of OFMSW in the feed, since peaks higher than 1.0 g N/kg were not observed
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23 571 from day 20 onwards, in contrast to mono-digestion reactors.
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32 573 NH₃ inhibition was likely one of the main triggers of overloading in this study, since the
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34 574 high NH₃ levels observed (i.e. ≥ 1.0 g N/kg) are normally associated with methanogenic
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36 575 inhibition and VFA accumulation in AD (Drosg, 2013; Rajagopal et al., 2013). Thus,
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38 576 despite each AD system might show particular NH₃ inhibition thresholds depending on
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40 577 the anaerobic consortia (Fricke et al., 2007; Westerholm et al., 2016), a gradual
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42 578 methanogenic adaptation to high levels of TAN (i.e. ≥ 4.0 g N/kg) might be crucial to
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44 579 increase OLR in semi-continuous HS-AD of OFMSW (Hartmann & Ahring, 2006;
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46 580 Rajagopal et al., 2013).
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53 582 In this study, a tradeoff was needed between the ‘undesired’ TAN buildup and the rapid
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55 583 TS removal observed, to reach HS-AD conditions (i.e. TS ≥ 10 %) with mono-digestion
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1 584 of OFMSW. For example, the different TS and TAN dynamics can be appreciated in
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3 585 mono-digestion reactor A from day 30, when TS fluctuated while TAN steadily
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5 586 increased [Figure 2]. Potential ammonia contingency strategies in AD, as increasing the
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7 587 substrate dilution, reducing the OLR, and/or increasing the MRT (Kayhanian, 1999;
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9 588 Rajagopal et al., 2013), would have lengthened considerably the experimental time, or
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11 589 even prevented to achieve HS-AD conditions (i.e. $TS \geq 10\%$) with mono-digestion of
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13 590 OFMSW.
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23 593 **4 CONCLUSIONS**

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25 594 In this study, reducing the effluent compared to the influent mass (i.e. 18 %) permitted
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27 595 to extend the MRT in semi-continuous mono-digestion of OFMSW, and obtain a
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29 596 specific biogas production of 229 L/kg VS added, due to the high biodegradability of
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31 597 OFMSW. However, the sole implementation of influent/effluent uncoupling was not
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33 598 sufficient to avoid reactor overload and acidification when reaching HS-AD conditions
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35 599 (i.e. $TS \geq 10\%$). The average OLR was 4.5 g VS/kg·d, whereas a maximum 11.5 % TS
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37 600 was reached. In contrast, the addition of beech sawdust to OFMSW allowed to operate
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39 601 co-digestion reactors with an average OLR of 8.3 g VS/kg·d, and reach a maximum
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41 602 29.0 % TS. Co-digestion lowered by 22 % the TAN content, though an average 186
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43 603 L/kg VS added of biogas was obtained. Therefore, the addition of sawdust, as an
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45 604 example of lignocellulosic substrate, to OFMSW (i.e. 1-2 g VS-Sawdust/g VS-
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47 605 OFMSW) is an adequate strategy to stabilize HS-AD at very high TS contents (i.e. 20-
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49 606 30 %). Nonetheless, a compromise must be found between increasing the TS content
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1 607 and reducing the specific biogas production by co-digestion, since both aspects strongly
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3 608 determine the HS-AD economy for OFMSW treatment.
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11 715 **TABLE AND FIGURE CAPTIONS**

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14 716 **Table 1:** Bio-physical-chemical characterization of substrates.
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16 718 **Table 2:** Physical-chemical characterization of inoculums.
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19 721 **Figure 1:** Experimental setup. 1) Reactor body; 2) reactor head; 3) feeding port; 4) gas
20 722 output; 5) gas measuring port; and 6) opening valves.
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22 724 **Figure 2:** Mono-digestion of OFMSW: a) Organic loading rate; b) mass retention time;
23 725 c) total solids; d) total and free ammonia nitrogen (NH₃); e) cumulative biogas
24 726 production; and f) methane content. Black arrows represent the NaHCO₃ addition in
25 727 reactor A, while dotted arrows represent the FeCl₂ or inoculum addition in reactor B.
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27 729 **Figure 3:** Mono-digestion of OFMSW: Volatile fatty acids and pH in a) reactor A; and
28 730 b) reactor B. Black arrows represent the NaHCO₃ addition in reactor A, while dotted
29 731 arrows represent the FeCl₂ or inoculum addition in reactor B.
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31 733 **Figure 4:** Co-digestion of OFMSW and sawdust: a) Organic loading rate – parentheses
32 734 indicate the sole addition of OFMSW; b) mass retention time; c) total solids; d) total
33 735 and free ammonia nitrogen (NH₃); e) cumulative biogas production; and f) methane
34 736 content.
35 737

36 738 **Figure 5:** Co-digestion of OFMSW and sawdust: Volatile fatty acids and pH for a)
37 739 reactor A; b) reactor B; and c) reactor C.
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Figure 1

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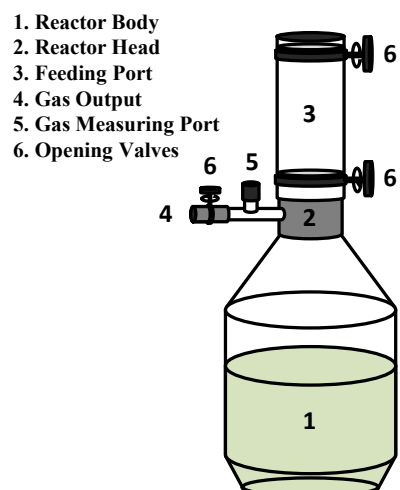


Figure 1: Experimental setup. 1) Reactor body; 2) reactor head; 3) feeding port; 4) gas output; 5) gas measuring port; and 6) opening valves.

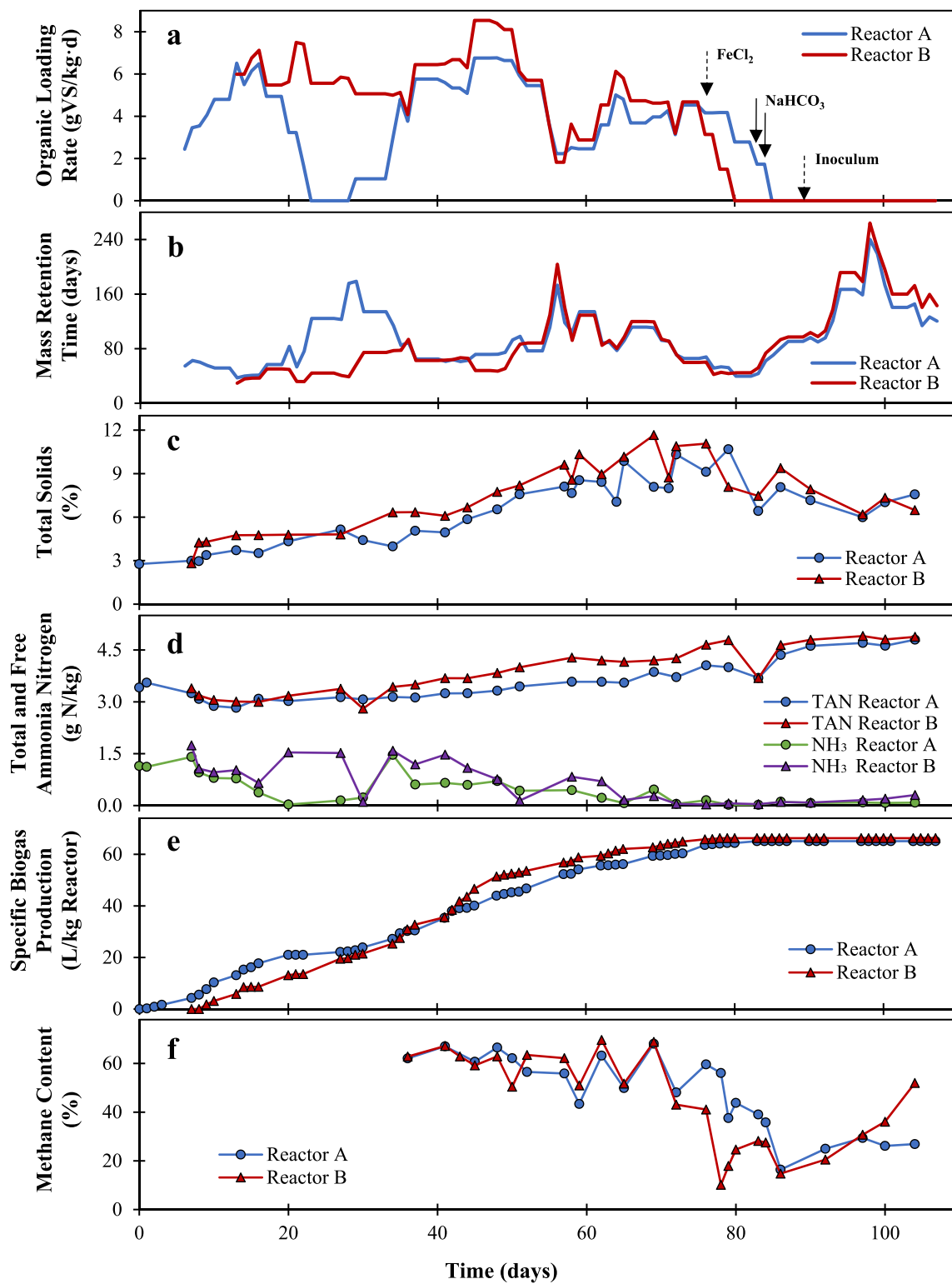
Figure 2[Click here to download Figure: Figure 2_corrected.pdf](#)

Figure 2: Mono-digestion of OFMSW: a) Organic loading rate; b) mass retention time; c) total solids; d) total and free ammonia nitrogen (NH_3); e) cumulative biogas production; and f) methane content. Black arrows represent the NaHCO_3 addition in reactor A, while dotted arrows represent the FeCl_2 or inoculum addition in reactor B.

Figure 3

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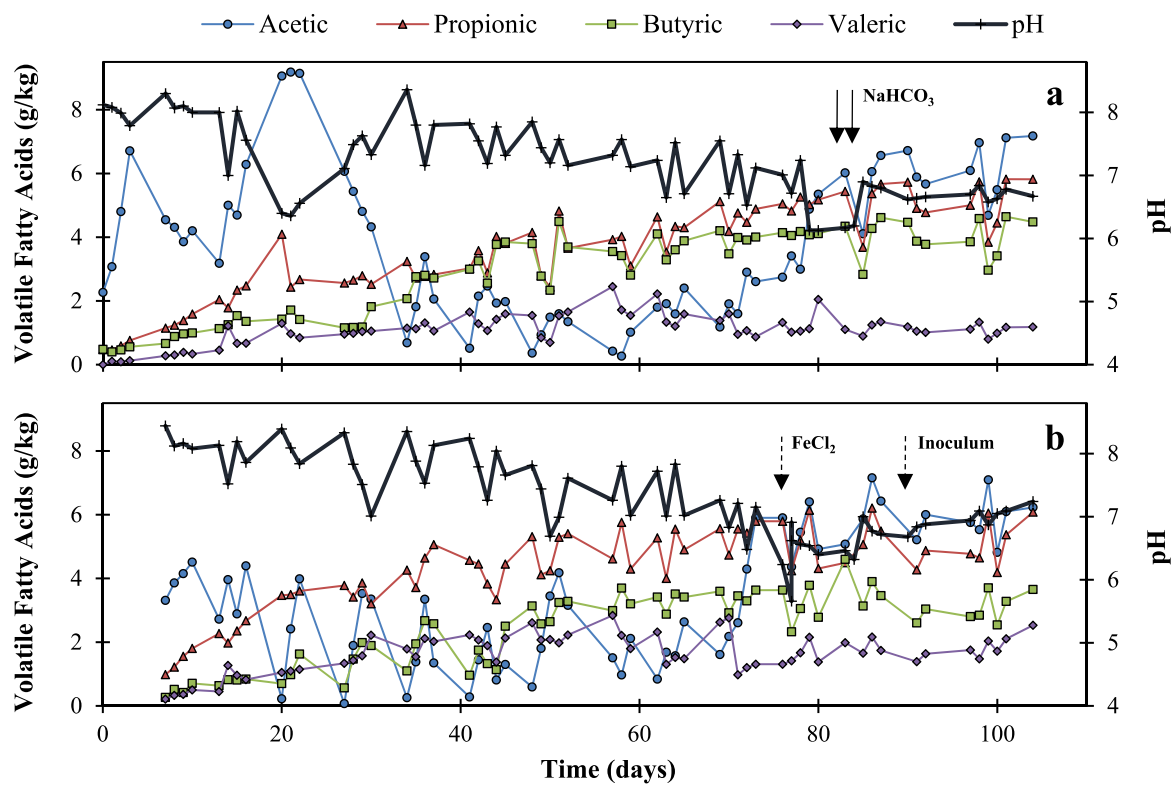


Figure 3: Mono-digestion of OFMSW: Volatile fatty acids and pH in a) reactor A; and b) reactor B. Black arrows represent the NaHCO₃ addition in reactor A, while dotted arrows represent the FeCl₂ or inoculum addition in reactor B.

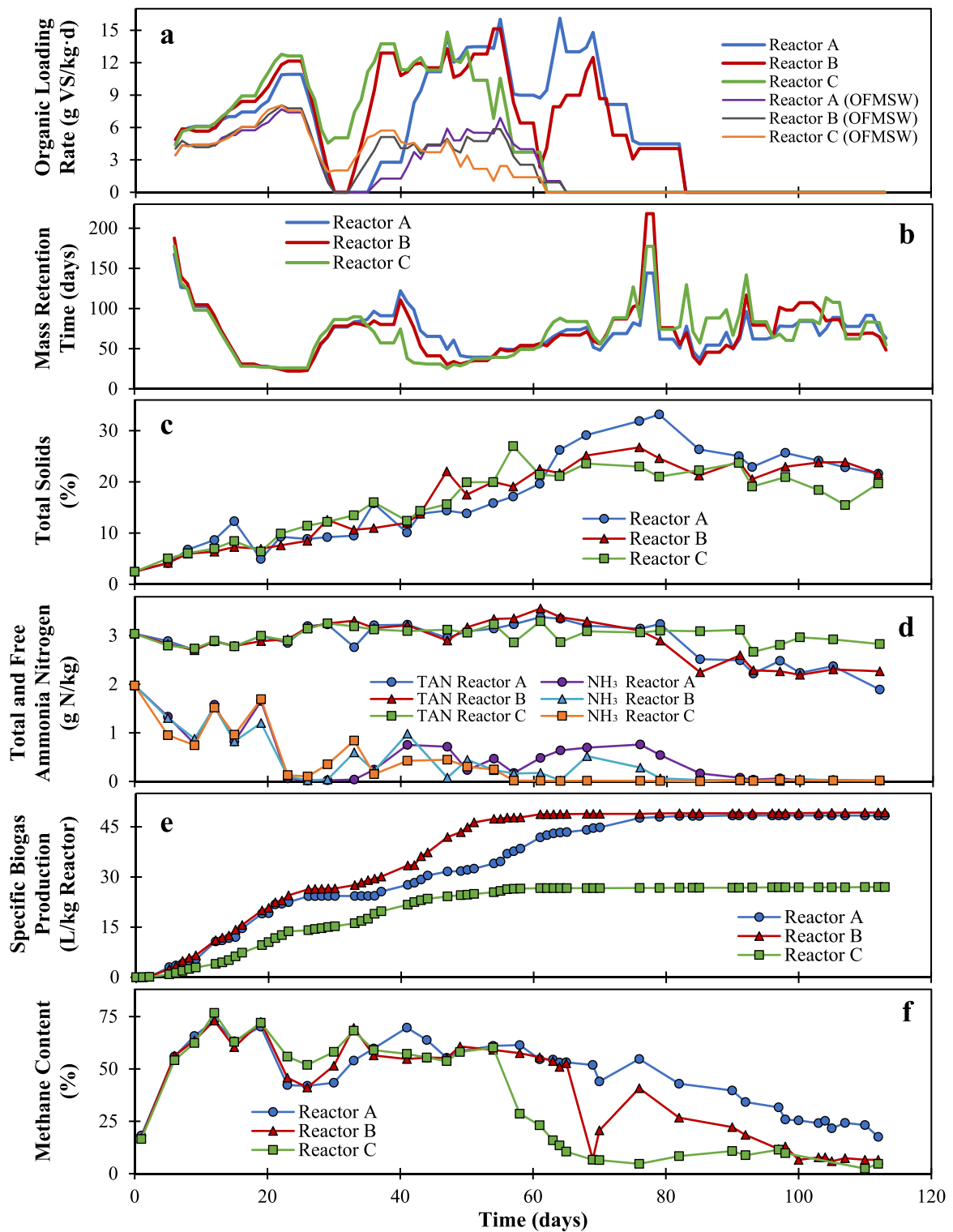
Figure 4[Click here to download Figure: Figure 4_corrected.pdf](#)

Figure 4: Co-digestion of OFMSW and sawdust: a) Organic loading rate – parentheses indicate the sole addition of OFMSW; b) mass retention time; c) total solids; d) total and free ammonia nitrogen (NH₃); e) cumulative biogas production; and f) methane content.

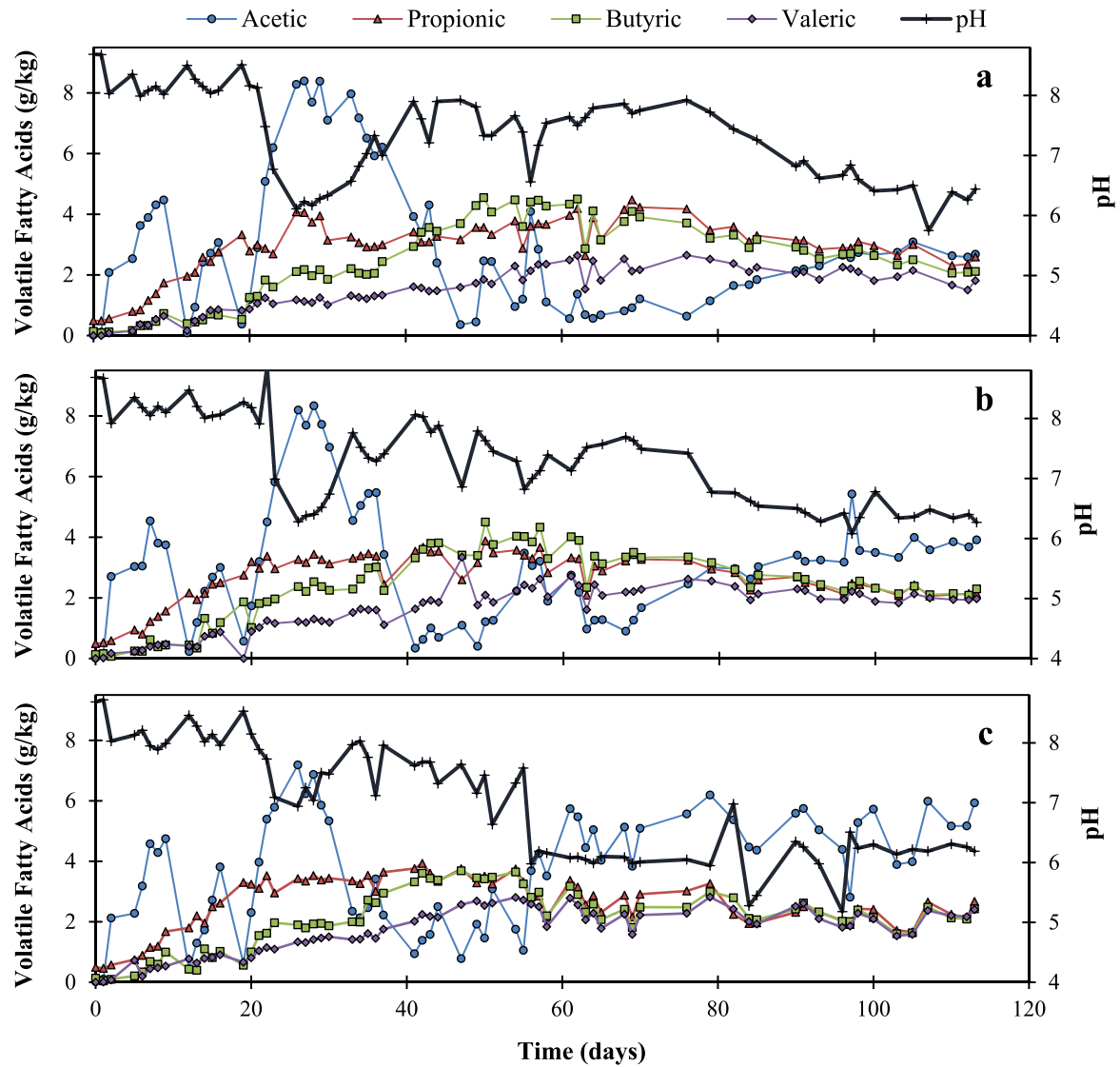


Figure 5: Co-digestion of OFMSW and sawdust: Volatile fatty acids and pH for a) reactor A; b) reactor B; and c) reactor C.

Table 1: Bio-physical-chemical characterization of substrates.

	OFMSW	Sawdust
TS (%)	26.52 ± 1.35	93.69 ± 0.42
VS (%)	24.62 ± 1.27	92.64 ± 0.70
VS/TS	0.93 ± 0.02	0.99 ± 0.01
TKN (g N/kg TS)	24.78 ± 1.50	0.98 ± 0.17
TAN (g N/kg TS)	4.92 ± 0.06	0.12 ± 0.01
pH	4.40 ± 0.14	5.65 ± 0.06
ALK_I (g Acetic/kg)	1.17 ± 0.82	1.50 ± 0.26
BMP (NmL CH₄/g VS)	497 ± 58	161 ± 12

Table 2: Physical-chemical characterization of inoculums.

	Mono-digestion		Co-digestion
	Reactor A	Reactor B	Reactors A, B & C
TS (%)	2.8	2.8	2.5
VS (%)	1.9	2	1.6
VS/TS	0.69	0.70	0.64
TKN (g N/kg TS)	161	161	139
TAN (g N/kg TS)	122	121	122
pH	8.12	8.44	8.69
ALK_P (g CaCO₃/kg)	9.6	9.6	9.3
ALK_I (g Acetic/kg)	5.3	6.3	3.2
Acetic (mg/kg)	2260	3310	20
Propionic (mg/kg)	470	980	490
Butyric (mg/kg)	480	260	140
Valeric (mg/kg)	0	210	0

Supplementary Information

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