‘Steam explosion as a pretreatment method to improve biogas production from wheat straw’

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I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Fokion Kaldis
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To my family……

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Abstract

Wheat straw (WS) is a lignocellulosic residue with high biogas potential. However, the composition and the crystalline structure of this residue is the main reasons for its low anaerobic biodegradability.

As a method to increase the biomethanation of WS, autoclaving, was conducted with a retention time of 30, 60 and 90 min. The subsequent digestion was conducted on a batch mode at different organic load rates (OLR) (2, 4, 8, 12 kg VS/m^3). The 60 minutes pretreatment seemed to be superior to all other tested treatments offering the highest enhancement on the biomethane production. Besides, for every OLR assessed, the addition of the liquid fraction generated during the pretreatment was found as inhibitory to the methane production. The inhibition effect was decreased along with the increase in the OLR.

Furthermore, a biomethane potential test (BMP) was conducted to evaluate the effect of steam explosion pretreatment, which conducted under 13 different pretreatment conditions, on the enhancement of the anaerobic biodegradability of WS. The severity factor (SF) for the pretreatment ranged between 2.61 and 3.35. The highest biomethane yields were offered from samples treated under SF of 2.76, 2.9 and 3.05. As a result, no clear conclusions can be made regarding the optimisation of the pretreatment conditions.

The steam explosion pretreatment of WS was also evaluated in a continuous AD system at two different OLR (2 and 5 g VS/L day^{-1}). According to the results from this experiment, for both the examined OLR, the steam-exploded samples offered increased methane yields on an average percentage of 20% compared to the yields produced after the digestion WS. During the same experiment, the composition and possible changes

in the microbial populations were monitored throughout the experimental period for both feedstocks. The identified microbial populations were similar for the digesters fed with WS and steam-exploded straw (SE). For both systems when the OLR was set to 2gVS/l the most dominant order was *Clostridiales* while after the increase of the OLR to 5 gVS/l the most abundant order was found to be *Bacteroidales.*

A combination and comparison between the steam explosion and a mechanical pretreatment were also evaluated. On a batch digestion mode, both pretreatments increased the produced gas yields while the combination of the two did not provide any significant enhancement. Similarly, for the continuous digestion mode experiment, the steam-exploded feedstock achieved higher gas yields compared to the mechanically pretreated ones. The two reactors digesting steam-exploded straw demonstrated higher instability with fluctuations on the pH values, fast accumulation of VFAs and low buffering capacity but they seemed to recover fast after the addition of buffer solution and a low-level re-inoculation. Regarding the effect of the two pretreatments on the microbial populations of AD, the steam explosion seemed to affect the microbiology of the system to a higher extent compared to the mechanical pretreatment.

In this study it was also evaluated the adjustment of the high C/N that WS usually has, with the use of NH_4Cl, combined with the steam explosion pretreatment as a method to further enhance the biogas yields from WS. A co-digestion of WS in various ratios with protein-rich food processing by-products [dried distillers' grains with solubles (DDGS) and rapeseed meal (RM)] was evaluated. According to the results of this experiment, the addition of NH_4Cl was more beneficial for the steam-exploded rather than the untreated WS. For the co-digestion of WS and SE with DDGS and RM, an increase in the cumulative methane production was noted when higher amounts of DDGS and RM

were added. On the other hand, the biodegradation of WS and SE was higher when lower amounts of food processing by-products were co-digested in the system.

Overall this work increases the knowledge on the steam explosion pretreatment of WS prior to AD and proposes additional techniques and methods for increasing the biodegradability of this type of biomass. According to the results, WS has potential for commercialisation as an AD feedstock while it can partially replace traditional AD feedstocks and potentially increase the financial and environmental sustainability of a full-scale biogas plant.

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1 Literature Review

1.1 The role of anaerobic digestion on the protection of the environment

During the last decades, biodegradable carbon recycling became one of the greatest challenges for the scientific community worldwide. In order to achieve future sustainability, there are many challenges ahead including environmental pollution, energy production in accordance with the replacement of the traditional fossil fuels, deforestation, climate change and other important issues. At the same time, controlling the utilisation of natural resources has emerged as a top priority related to problems such as global warming and the depletion of natural resources. In that direction, a strategy known as the circular economy is currently promoted by the European Union and several countries including China, Japan, Canada, UK, and many more (Korhonen et al., 2018). A circular economy could offer realistic solutions for modern societies and contribute to the development of sustainable approaches towards waste management and the exploitation of substances that are currently considered as waste. Based on this approach, resources are in use for as long as possible, while the use of non-renewable natural resources is decreased (Korhonen et al., 2018). Following that direction, the European Union has set a target of replacing an average of 40 % of the traditional fossil fuels used in 1990 with alternatives from renewable sources by 2030 (European Commission, 2018). Anaerobic digestion (AD) is a biological process aligned with both approaches (e.g. the production of clean bioenergy and the circular economy) as it provides renewable fuel (biogas) through organic waste treatment. AD at the same time increases the utilisation of underutilised material/feedstock and it incentivises the circularity by increasing the lifecycle of the feedstock. Furthermore,

the potential replacement of traditional chemical fertilizers by AD digestate also promotes the idea of the circular economy (Czekała et al., 2020).

From a general point of view, anaerobic digestion is a naturally occurring process, often encountered in the digestive system of animals or at the bottom of lakes where oxygen is absent (Ward et al., 2008). During the AD process, a consortium of anaerobic and facultative anaerobic microorganisms (e.g. bacteria and archaebacteria) cooperate under conditions of absence of oxygen and biogas is the main product. Biogas mainly comprises carbon dioxide (CO_2) and methane (CH_4). The percentage (%, v/v) of CO_2 and CH_4 in biogas range between 50–75% and 25–50%, respectively. Lower concentrations of other elements such as hydrogen sulphide (H_2S), ammonia (NH_3) and trace concentrations of hydrogen (H_2) and carbon monoxide (CO) can also be detected in biogas (Frigon and Guiot, 2010) (Table 1-1).

Table 1-1: Chemical composition of biogas, adapted by Frigon and Guiot (2010)

Components	Percentage (v/v)
Methane (CH_4)	50–75%
Carbon dioxide (CO_2)	25-50%
Hydrogen sulphide (H_2S)	1–2%
Nitrogen (N_2)	0–1%
Hydrogen (H_2)	0–1%
Carbon monoxide (CO)	Traces
Oxygen (O_2)	Traces

AD is widely applied for industrial purposes to manage heterogeneous waste such as food waste or municipal and industrial wastes and wastewaters (Koch et al., 2015; Zarkadas et al., 2016) and/or to produce bioenergy. During the AD process, the degradable components present in a substrate in a non-utilisable form, such as cellulose, is transformed by microorganisms into renewable fuel, valuable nutrients and other macromolecules. At the same time, the use of biomass as a renewable energy source from AD systems outperforms other strategies towards the production of 'green' energy, such as the use of photovoltaic accumulators or windmills. The main advantage of the AD process compared to other renewable energy technologies is its independence from meteorological conditions (Theuretzbacher et al., 2015b). The reduction of volatile solids (VS) content of the substrate by the microbial consortia during AD, is directly associated with the final product of the process (biogas). Furthermore, the use of AD for treating organic wastes can ideally decrease the volume of materials that end up in landfill, improving as a consequence the air quality around those areas by decreasing odours that organic solid wastes produce (Hilkiah Igoni et al., 2008). In addition, the replacement of fossil fuels with biogas can also contribute to the improvement of air quality by decreasing particulate matter emissions. Finally, apart from biogas and the change of status of the feedstock from waste to resource, the AD process can also offer further benefits in the production of agricultural products in rural areas. More specifically, the effluent sludge (digestate) of an AD bioreactor has high nutrient content in nitrogen (N), phosphorous (P) and potassium (K) and can be spread and used as a bio-fertiliser into fields (Wrap, 2012).

1.2 Stages of the anaerobic digestion

The AD process comprises four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The products of each stage serve as a substrate for the next one, while under optimum conditions of digestion, no accumulation of intermediate products takes place (Figure 1-1). The last step of the AD process (methanogenesis) comprises two different pathways; acetoclastic and hydrogenotrophic methanogenesis. During the acetoclastic methanogenesis, the acetic acid produced during the previous stages of the process is converted into carbon dioxide and methane by specific methanogenic archaebacteria, including the strictly acetoclastic methanogen *Methanosaeta sp* (Qu et al., 2009). On the other hand, hydrogenotrophic methanogenic archaea utilise CO_2 and H_2 in order to produce CH_4. Usually, the two pathways for the production of methane can co-exist inside an anaerobic bioreactor and their efficiency can change over a digestion period based on environmental conditions of the reactor (temperature, presence of VFAs) (Qu et al., 2009).

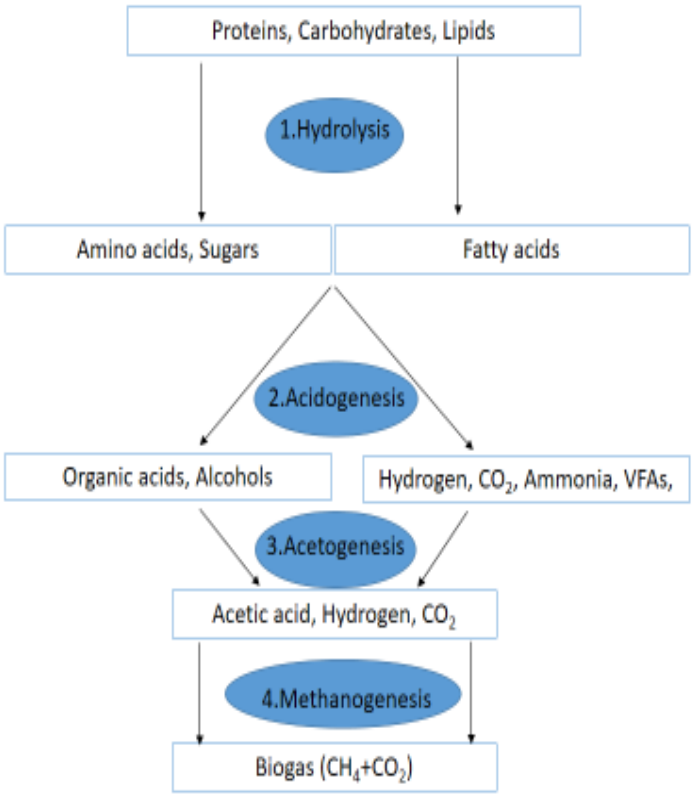

Figure 1-1: Schematic illustration of the four different stages of anaerobic digestion system (adapted from Prakash et al., (2015))

1.3 Commercial uses of biogas

Modern societies can take advantage of biogas production by using it as a renewable and cheap alternative fuel to several processes that are required on a daily basis. Biogas can be used directly as a feed to internal combustion engines and fuelling systems including boilers and turbines, towards the production of mechanical work and/or heat, electricity, warm water and others. At the same time, biogas can be used as fuel for transportation vehicles. Over the last two decades, a continuous increase in the use of biogas as vehicle fuel has been observed. As an example, the expected annual consumption of biogas as a vehicle fuel in Sweden for 2020 is expected to reach 1 TWh, when back in 2002 it was measured to be 100 GWh (Haider et al., 2018).

1.4 Biogas upgrade

A modern approach related to biogas production and exploitation is called biogas upgrade. Upgraded biogas has similar characteristics to natural gas, while its CO_2 content must be below the limit of 6% according to regulations for European countries (Meier et al., 2019). The idea behind this technology is that after the removal of the CO_2 that is present in biogas, the remaining gas, which is also known as biomethane, can be used as a substitute for natural gas while it can be directly injected into the natural gas pipelines. Currently, the main technologies of biogas upgrade are based on chemical or physicochemical processes. Morero et al., (2017) compared different types of solvents for the production of higher quality biogas with the use of an absorber-stripper process. Another proposed option for biogas upgrade is the pressure swing adsorption system. The use of this technology requires the additional use of adsorbents such as activated carbon, silica gel or zeolite 13X (Ferella et al., 2017). Furthermore, another alternative that has already been used on a commercial scale is the use of membranes (Peppers et al., 2019). Unfortunately, the cost of all the above technologies is still relatively high, in addition to the extra need for managing chemicals and produced wastes. In contrast to the physicochemical methods which are commercially used today, the biological biogas upgrade has been proposed as a promising alternative (Bassani et al., 2016; Treu et al., 2018). During this process, an extra amount of H_2 can be injected into the biogas tank and subsequently this could be used as an electron donor by the hydrogenotrophic methanogenic archaea which utilise carbon dioxide as a carbon source (Bassani et al., 2016). The required H_2 can be found in the surplus energy that is produced from windmills or photovoltaic facilities. More

specifically, this extra amount of the already produced energy, which t would be produced on a high wind season, can offer, through water electrolysis, significant amounts of H_2. This amount of H_2 can subsequently be used in order to enhance the hydrogenotrophic methanogenesis (Bassani et al., 2016). Biogas upgrade can happen either *in situ* or *ex-situ* in an anaerobic bioreactor (Voelklein et al., 2019). Although the biogas upgrade seems to be a very attractive approach for the AD industry, additional research is required to maximise the positive outcomes of the process and at the same time to minimise the obstacles.

1.5 Additional by-products of anaerobic digestion

Apart from a 'green' and renewable source of energy, anaerobic digestion has the potential to offer more products towards the creation of a sustainable future for the planet. Among others, the AD process generates high quality biological and nutrient-rich fertilizer, also known as digestate. The utilisation of digestate as a fertiliser offers multiple benefits towards the economic and environmental sustainability of a full-scale biogas plant. It should be mentioned that for a company that feeds its AD system with its homegrown energy crops, no additional source of chemical fertilizer is required, as the digestate can also act as a soil improver. At the same time, by using digestate as the main soil fertilizer, nutrients from the bioreactor will return to the natural environment closing in that way their cycle which is in line with the idea of circular economy (Czekała et al., 2020). In that direction, the profitability of the AD process can also be increased (Czekała et al., 2020). Except for digestate, AD can also offer some valuable intermediate chemical by-products, such as volatile fatty acids (VFAs) (Scoma et al., 2016), which can be further used in several applications. As an example, VFAs can be

used as building blocks for the production of biodegradable polyhydroxyalkanoates (PHAs), which can subsequently be used to replace traditional plastics (Lagoa-Costa et al., 2017).

1.6 Different feedstocks used in anaerobic digestion systems

Anaerobic digestion is a well-studied process where almost every organic material, including organic waste such as municipal and industrial wastewaters or solids waste, can be used as a potential feedstock when specific digestion criteria are met. Examples of already proposed and tested AD feedstocks include, but are not limited to, animal manure (Kafle and Chen, 2016), food waste (Capson-Tojo et al., 2016), microalgae and macroalgae (Rodriguez et al., 2015), wastes and wastewaters from different agro-industrial processes (Rodríguez-Abalde et al., 2016; Zarkadas et al., 2019), paper and paper pulp (Veluchamy and Kalamdhad, 2017) and several lignocellulosic residues (Cysneiros et al., 2012; Tsapekos et al., 2018). Apart from the mono-digestion process, an alternative strategy is co-digestion, where feedstock with differences in their composition are digested together inside the bioreactor aiming an enhanced biodegradability of all the under treatment material (Koch et al., 2015; Risberg et al., 2013; Rodríguez-Abalde et al., 2016). The principle of this is the improvement of the digestion conditions inside the bioreactor, through either diluting difficult to digest materials or by providing characteristics to the system that a sole substrate cannot offer. For instance, when the pH of the system drops to levels outside the acceptable for AD microorganisms, the addition of a substrate with pH on the alkali range can prevent the collapse of the system by balancing the pH and at the same time offer increased biogas yields.

1.7 Lignocellulosic feedstocks in anaerobic digestion

1.7.1 Energy crops

As the market of renewable energy is rapidly developing, biogas is also gaining increased interest. In Finland for example, biogas production doubled between 2011 and 2017 (Winquista et al., 2019). Throughout the world, a wide variety of full-scale biogas plants operate with the use of energy crops as feedstock (Naegele et al., 2012). At the same time, prices of traditional AD feedstocks are fluctuating based on different reasons including the weather conditions that affect the energy crops production. Unfortunately, the future of AD technology on an industrial scale heavily relies on the use and improvement of gas production from alternative and cheap feedstocks. The lignocellulosic residues (or lignocellulose) refers to plant-based dry material which is also known as biomass. The biofuels that have been produced with energy crops as biomass feedstock source are categorised as first-generation biomass energy (Almeida Streitwieser, 2017) while the as second-generation biofuel, or cellulosic-based biofuel, can be considered the ones coming from non-edible matter from crops. Although energy crops usually offer a satisfactory biomethane recovery due to their high content in easily degradable sugars, their digestion is also associated with some disadvantages. First, the cost for the cultivation of feedstock such as corn maize increases the final price for the subsequent bioenergy production. Furthermore, the cultivation of energy crops can potentially come into direct competition with products that are intended for human consumption or animal feed, for the use of the same arable land. As a consequence, the prices of the food products are increasing, leading potentially to a shortage of food (Kesharwani et al., 2019).

1.7.2 Lignocellulosic wastes

Lignocellulosic waste represents low-value biomass that is left behind after harvesting. Wheat straw (WS), which is the stalk left in the field after harvesting the wheat grains, represents a characteristic example of lignocellulosic waste biomass that can be used for biogas production (Kesharwani et al., 2019). The use of lignocellulosic waste as feedstock for bioenergy production not only generates a considerable amount of renewable energy but also contributes to the waste management of those materials, which would otherwise be used as a bedding material for ruminants, left unused or even burnt in the fields (Chandra et al., 2012b).

1.8 Composition of lignocellulosic residues

Lignocellulose mainly comprises of carbohydrate polymers, in the form of cellulose and hemicellulose, as well as lignin, a complex aromatic polymer. Lignocellulosic residues also present smaller amounts of extractives and ash (Han et al., 2009).

1.8.1 Cellulose

Cellulose is a hydrophilic, insoluble organic polymer comprising of several hundred to many thousands $\beta_{(1\rightarrow4)}$ linked D-glucose units, in a linear chain (Tian et al., 2018). Cellulose macromolecules contain several hydroxyl groups which form a plethora of intra- and intermolecular hydrogen bonds. This results in the creation of various ordered crystalline arrangements (Park et al., 2010). However, cellulose fibres of lignocellulosic biomass consist of both crystalline and amorphous regions. The ratio between those values is referred to as the crystallinity index (CI) and depends on the type of lignocellulose, the period of harvesting and other environmental conditions. The crystalline regions are more ordered while the amorphous is not intact (Park et al., 2010). In general, a material with a higher content of amorphous regions is more sensitive to biological and chemical attack because of their increased surface area which allows the microorganisms to colonise it (Rajput et al., 2018). The amorphous regions can also offer increased accessibility to the enzyme's cellulase active sites (Tian et al., 2018). As a result, a material with a higher crystallinity index can be assumed as a more favourable feedstock for AD systems.

1.8.2 Hemicellulose

Hemicellulose is another organic polysaccharide which, unlike cellulose, is amorphous in nature and consists of a variety of different carbohydrates including pentoses as well as hexoses, together with some acids, such as galacturonic acid (Kainthola et al., 2019). Hemicellulose along with lignin acts as a physical barrier to microbial and enzymatic attack to cellulose. Prior to the AD process, carefully chosen pre-treatments can enhance the solubility of the hemicellulose fraction into simpler sugars, allowing the hydrolysis of cellulose and hemicellulose, enhancing in that way biogas production.

1.8.3 Lignin

Finally, lignocellulosic residues present a high content of the difficult anaerobically degradable lignin. Lignin is a complex polymer that creates a physical protecting seal around both cellulose and hemicellulose (Zhou et al., 2017). A high presence of lignin along with low anaerobic biodegradability of lignocellulosic residues, mostly due to their structure, renders pretreatment as a stage required for further digestion (Mancini et al., 2018). A previous study assessed 90 different varieties of wheat straw (WS) and reported differences in their composition and chemistry depending on the height of the plant, the stem proportion and the leaf tissue (Collins et al., 2014). Those changes can potentially cause variations in the anaerobic biodegradability of different WS samples.

1.9 Wheat straw as feedstock to anaerobic digestion

Different lignocellulosic residues have been examined as a possible feedstock for AD systems. Residues with a good biomethane potential include meadow grass (Tsapekos et al., 2015), rapeseed straw (López-linares et al., 2015), maze straw (Hua et al., 2016)

and rice straw (Candia-García et al., 2018; Zealand et al., 2017). Apart from the above, a very promising lignocellulosic feedstock for AD is WS. WS represents one of the most studied material regarding its biogas production. First of all, its high availability in accordance with its usually low prices renders this residue a financially affordable material for industrial use as feedstock towards bioenergy production. However, it should be noted that continuous removal of straw from the fields in order to produce bioenergy has a potential on the decrease in the fertility of the soil, as a consequence of depletion of the soil organic carbon and the inevitable soil erosion (Peng et al., 2016). However, the return of the AD produced digestate in the fields can act as a natural replacement for straw.

Over the years, several parameters of the AD of WS have been examined by the scientific community aiming at the enhancement of biogas production from this residue. Even though WS has a high theoretical biomethane production, usually its anaerobic biodegradability is low. The main reasons for this are the high lignin content compared to other lignocellulosic biomasses, as well as the high C/N ratio which usually presents values higher than 80:1 (w/w). For a successful biodegradation process with respect to the C/N ratio, an exact balance of nitrogen and carbon is needed to cover the nutritional needs of the microorganisms. On the other hand, excessive amounts of N_2 can cause an inhibition of the process. A high C/N ratio can lead to rapid consumption of nitrogen by the microorganisms and suboptimal production of biogas due to acidification, poor buffering capacity and the possibility of high volatile fatty acids (VFA) accumulation from biodegradation of the carbon source (Banks and Humphreys, 1998). In such occasions where low pH occurs inside the anaerobic bioreactor, the population that faces the highest reduction in their numbers are the methanogenic Archaebacteria as they are more sensitive to pH fluctuations according to Jiang et al., (2019). In contrast,

low C/N ratio values of the feedstock can result in ammonia accumulation and high pH values inside the anaerobic bioreactor, which can render the process toxic to the microorganisms. In general, studies have proved that AD microorganisms consume available carbon approximately 30-35 times faster than nitrogen. According to Hilkiah Igoni et al., (2008) a C/N ratio of 30:1 is optimal for the AD process. In that direction and in order to adjust the C/N to the optimal range, many researchers have proposed to co-digest WS with material with a high presence of nitrogen. Hassan et al., (2016), examined the synergetic effect of the addition of wheat straw (WS) and chicken manure (CM) on four different C/N ratios (35:1, 30:1, 25:1 and 20:1) while he identified the ratios between 20:1 and 30:1 as the most effective. Furthermore, apart from the imbalanced C/N, the rigid structure of this residue plays a significant role in the usually decreased efficiency of the whole process towards the production of biogas (Rajput et al., 2018). In order to tackle this, a pretreatment stage is usually applied to the feedstock prior to AD (Yu et al., 2019)

1.10 Operational requirements of anaerobic digestion

Similar to all biological systems, AD has some minimum requirements in order to work under steady-state and produce significant volumes of gas. During the design of AD systems, parameters such as the pH, organic loading rate (OLR), hydraulic retention time (HRT), temperature, solids content inside the bioreactor, C/N ratio, availability of the system to nutrients and micronutrients, production of intermediate by-products during the process and the presence of potential inhibitors must be taken into consideration. In the AD process, all operational parameters are connected and failure

to meet the appropriate standards for one can be the basis for an inhibition effect which can eventually cause a total failure of the system.

1.11 Parameters affecting the anaerobic digestion of lignocellulosic wastes

1.11.1 Temperature

Temperature is an important parameter that affects the AD of lignocellulosic residues, such as WS. Two of the most well-examined temperature values in AD systems are the mesophilic temperatures (38 ± 2 °C) and the thermophilic temperatures (55 ± 2 °C). An important difference in AD systems operating at those two temperature values is the improved biogas production of thermophilic digestion compared to that of the mesophilic systems (Heeg et al., 2014). The same study also revealed that temperature changes caused alternations in the microbial population inside AD bioreactors with the largest difference reported for the population of the methanogenic archaebacteria. On a thermophilic system, the methanogenic activity is higher and this contributes to a faster breakdown of the feedstock (Fountoulakis et al., 2008). Although in most cases the thermophilic anaerobic digestion seems to be more efficient compared to the mesophilic, the extra cost of increasing the digestion temperature needs to be taken into consideration when an AD system is designed. Moreover, the enhanced hydrolytic activity that thermophilic digestion offers compared to mesophilic conditions can result in quicker and greater accumulation of intermediate by-products (e.g. VFAs). High accumulation of VFAs inside an AD bioreactor can act as a growth inhibitor for the total methanogenic population of the system (De Francisci et al., 2015). Nevertheless, examples of bioreactors operating at a lower range of temperatures have also been reported for the digestion of WS with cow manure (Massé et al., 2015). This last system is called psychrophilic AD and works at temperatures close to 20°C (Saady and Massé,

2013). The four different stages of the AD process do not necessarily have the same optimal range for the temperature and therefore Ward et al. (2008) proposed that a multi-stage AD system could potentially offer the best outcomes if the acidogenic reactor operates at a different temperature rate than the methanogenic one.

1.11.2 The pH

Apart from the temperature, changes in the pH inside an anaerobic bioreactor can also affect the microbial population, including methanogenic archaebacteria, as well as the enzymes that take part in the biochemical reactions. A general optimal pH range for AD systems is between 6.0 and 8.3 but depends on the digestion type as well as the substrate that is utilised. Although the pH value inside the bioreactor can slightly exceed those values (Liu et al., 2008), a pH level below this range can inhibit methanogenic activity, while an extremely alkaline pH can cause cell lysis of the microbial granules and as a result, the process can be highly inhibited. According to Angelidaki and Sanders (2004), most of the methanogenic archaebacteria have an optimal pH range between 7 and 8 while for acidogenic microorganisms, their optimal growth pH is often lower. Again, two-stage digestion systems have been proposed as a method to enhance the digestion process while the two separated bioreactors are working under different pH conditions (Cysneiros et al., 2011).

1.11.3 The hydraulic retention time (HRT)

An additional requirement that needs to be taken into account when an AD system is designed is the hydraulic retention time (HRT). HRT represents the average time that the AD treated material remains inside the bioreactor. HRT is a very important parameter since it affects the interaction of the feedstock with the microorganisms. Shi et al. (2017) examined three different HRT (20, 40 and 60 days) for the digestion of

WS on a continuous stirred-tank reactor (CSTR) system at mesophilic conditions. According to the results of this study, the HRT affects the stability of the process and as a consequence, it also affects biogas production. In the same study, it was also reported that the degradation of cellulose and hemicellulose was better when higher HRT was applied (Shi et al., 2017).

1.11.4 Organic Loading Rate (OLR)

The amount of feedstock added to the digester, either daily for a continuous/semi-continuous system or at the beginning of the process for a batch system, can be defined as organic loading (rate in the case of continuous systems; OLR) and plays an important role in the productivity of the AD process. Kaparaju et al. (2010) showed, in experiments where was a sole substrate for upflow anaerobic sludge blanket UASB reactors, that an increase in the OLR from 17.1 g COD/ ($l_{reactor}\ d^{-1}$) and at a substrate concentration of 25% in the feed to 41.2 g COD/ ($l_{reactor}\ d^{-1}$) and/or substrate concentration to 33–50%, has the potential to decrease the biomethane production up to zero values. The effect of OLR highly depends on the type of substrate used and other parameters such as the acclimation of the inoculum. For example, it has been found that in the case of easily degradable feedstock such as vegetable wastes, the optimal OLR was at 1.4 kg VS/ $m^3\ day^{-1}$ (Babaee and Shayegan, 2011) while for food wastes digestion an OLR of 9.2 kg VS/ $m^3\ day^{-1}$ was still within the acceptable for the process limits (Nagao et al., 2012).

1.11.5 Accumulation of Volatile Fatty Acids (VFAs)

The accumulation of intermediate products in the process of anaerobic digestion (e.g. volatile fatty acids, VFA's) is directly associated with the OLR that the system operates at. VFAs are short-chain fatty acids comprising of two to eight carbon atoms and can

be found in AD as products of acidogenesis and acetogenesis. WS contains a notable amount of easily degradable components and in combination with a high OLR, can result in rapid production of VFA and probably in the inhibition of the system (Rouches et al., 2019). The inhibition effect of the increased accumulation of VFAs will be higher in cases where the amount of methanogens in the system is low (Angelidaki and Sanders, 2004).

1.11.6 Availability of nutrients and the presence of inhibitors

Other parameters which need to be taken into account when an AD system is designed is the availability of micronutrients and the presence of some potential inhibitors in the system. The poor content of WS in both micro- and macronutrients is a significant drawback with regards to its biomethanation potential (Nges et al., 2015). For instance, there is evidence that the addition of an adequate amount of phosphate (465 mg-P/L) can increase the productivity of biogas from rice straw (Lei et al., 2010).

Inhibitors of AD can be components coming directly from the feedstock or by-products of the process itself. Additionally, inhibitors might be introduced as a result of pretreatment implemented prior to AD. As an example, a hydrothermal pretreatment of a lignocellulosic residue at severe conditions (e.g. 200 °C or higher) can potentially produce furanic compounds such as 5-HMF and furfural due to the dehydration of hexoses and pentose respectively, that are presented in the feedstock (Phuttaro et al., 2019). Both compounds can potentially cause an inhibition of the AD process when supplied in high concentrations. Another study has shown that the methanogenic Archaeabacterium, *Methanococcus sp*., strain B, grew better when furfural was present in concentrations up to a specific level, while the same system was inhibited when higher doses of furfural were present (Boopathy, 2009). Toxic compounds for the AD

process also include several organics such as pesticides and fumigates, heavy metals, ammonia, sulphide and other compounds (Chen et al., 2014).

1.12 Wet and dry anaerobic digestion

An AD system can also be characterised as either "wet" or "dry". The total solids content in the influent of the bioreactor is the parameter that categorises digestion wet when TS <15% (w/w), or dry when the TS>22% (w/w). When the TS content is in between 15 and 22% (w/w) the digestion can be characterised as semi-dry (Motte et al., 2013). Solid-state anaerobic bioreactors can potentially be used for the digestion of lignocellulosic residues due to the usually low moisture content of these residues (Ge et al., 2016). Solid-state anaerobic digestion (SSAD) can offer a reduction in the specific volume of the reactor, lower required input energy and an increase of the OLR (Motte et al., 2014). However, the efficiency of this method is generally lower compared to liquid-state anaerobic digestion (LSAD).

1.13 Pretreatments prior to the AD process

Almost every AD feedstock can potentially increase its biogas production potential after the use of a pretreatment stage before the main anaerobic digestion process. Usually, the main aim of the pretreatment is to render the digestible components of the feedstock more accessible for the microorganisms of the process and their enzymes. For example, in lignocellulosic residues, the easily degradable carbohydrates are well bonded with the non-degradable lignin which limits the efficiency of the production of biogas. Different pretreatment methods result in different effects on the subsequent digestion for the same type of feedstock. The microbial composition of the system, their growth kinetics, as well as the amount and the quality of the produced biogas, are only

some of the parameters that are directly connected with the pretreatment choice. The choice of the pretreatment strategy is highly depended on different parameters including the type of feedstock, the investment and operational costs for the equipment along with the cost for the consumables of the pretreatment and its environmental and financial sustainability. At the same time, by-products of the pretreatment process might need to be removed or be treated according to the legislation of the country where the pretreatment takes place. Moreover, a consideration of a potential scaling up from lab or pilot scale to industrial scale must be evaluated when a new pretreatment is tested or considered. For example, a microwave pretreatment might offer a significant increase in biogas production on a lab-scale (Qian et al., 2019) but its use on an industrial-scale might not be feasible due to economical restrictions. For lignocellulosic residues, the already examined methods include different chemical pretreatments such as the addition of alkalis or the organosolv method (Mancini et al., 2018; Romero-Güiza et al., 2017). Acids have also been tested as a pretreatment method with the scope of breaking of the ether bonds between lignin and the carbohydrates without dissolving lignin (Sambusiti et al., 2013). Biological methods include the use of fungi prior to the AD process (Rouches et al., 2019), as a tool to break down the lignin in the feedstock. Another well studied pretreatment choice for lignocellulosic residues is mechanical pretreatment. In this category belong processes that aim to achieve biomass deconstruction using shear and/or compression forces and include methods such as grinding or maceration (Dumas et al., 2015; L.C. Ferreira et al., 2014; Menardo et al., 2012). The decrease in particle size can offer increased efficiency during the mixing of the bioreactor and also result in increased accessibility of the microorganisms to the digestible components of a given feedstock (Tsapekos et al., 2015). Finally, a combination of the above pretreatment methods has been tested (Barakat et al., 2014;

Theuretzbacher et al., 2015a). Figure 1-2 summarises the pretreatment choices for lignocellulosic residues.

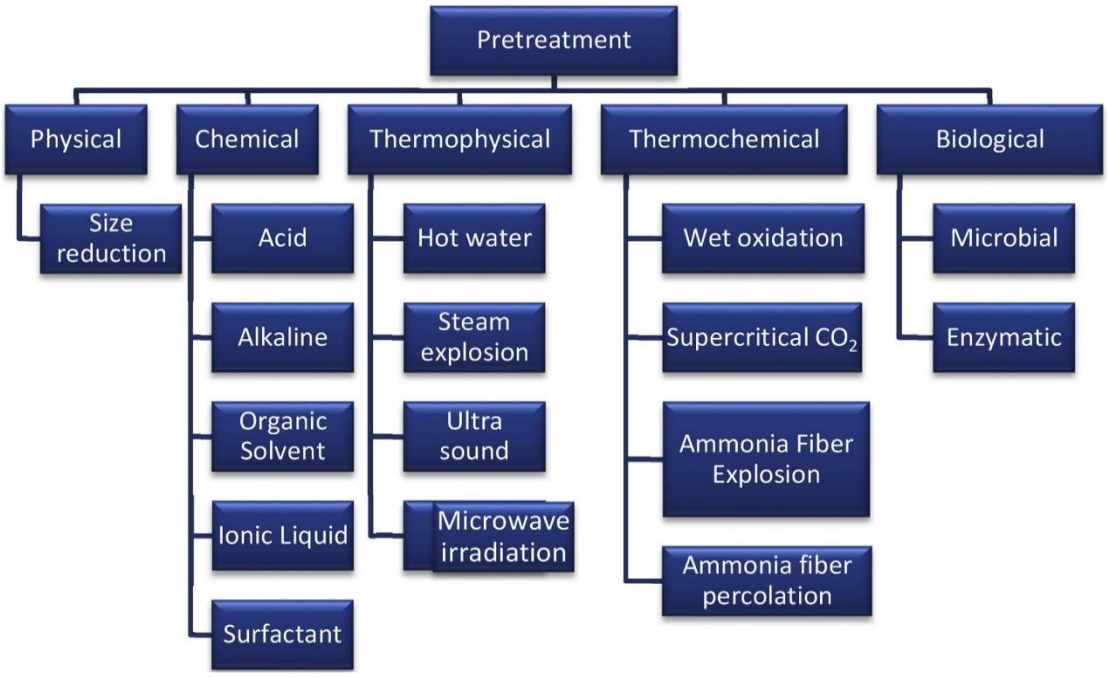

Figure 1-2: Pretreatment choices towards the improvement of biogas production from lignocellulosic residues. * Adapted from Kainthola et al., (2019)

1.13.1 Hydrothermal (steam explosion) pretreatments

In addition to the above techniques, hydrothermal pretreatment (including steam explosion) is one of the most common methods for the valorisation of lignocellulosic materials and the production of biofuels (López-linares et al., 2015; Tomás-pejó et al., 2017; Vaithanomsat et al., 2009) and biogas (Dereix et al., 2006; Estevez et al., 2012; L. C. Ferreira et al., 2014). However, the microbes taking part in the AD process present higher tolerance to inhibitors generated from the steam explosion, compared with the process of bioethanol and as a consequence, detoxification is less likely to be needed according to Zheng et al. (2014).

When a lignocellulosic material is subjected to a steam explosion pretreatment, it is placed into a closed vessel under conditions of high temperature (up to 240 °C) and pressure (up to 33.5 bar) for a specific pre-decided period (Bauer et al., 2009). After the end of the pre-set period, the pressure is discharged abruptly to atmospheric levels (1 atm). This release can theoretically cause changes in the structure and composition of biomass due to an explosive decompression that consequences in a rupture of the rigid structure (Li et al., 2016). As a result, hemicellulose is hydrolysed to simpler sugars and lignin is unwrapped from cellulose and hemicellulose, rendering them both more accessible for biological decomposition.

There is a significant number of studies in the international literature that have examined the effect of hydrothermal pretreatments on the anaerobic digestion of lignocellulosic biomass. Bauer and his team worked with a steam explosion pretreatment to enhance the biogas production of late-harvested hays (Bauer et al., 2014) while Estevez et al. (2014) used steam-exploded Salix as a feedstock for their AD experiments. The scientific teams of Menardo, Zhou and Chao Li chose Miscanthus for their experiment (Li et al., 2016; Menardo et al., 2013; Zhou et al., 2017), while more examples of tested residues are birch and reed (Lizasoain et al., 2016; Vivekanand et al., 2013). Straw is worldwide the most abundant type of lignocellulosic biomass, with wheat straw representing the most common type in Europe and the second most usual in the world after rice straw (Wang et al., 2009). Only in China, an amount of 130 million tons of WS were produced in 2016 (Liu et al., 2019). Based on these numbers and taking into consideration the relatively low price of this residue, many researchers have chosen to test the effect of hydrothermal pretreatments of different types of straw prior to its biomethanation. Zhou et al. (2016) and Theuretzbacher et al. (2015) reported increased biomethanation for steam-exploded

RW and WS respectively with the note that the energy production from these two materials can be combined with cereal grain with the main goal to be the increase of the profit of the whole process(Theuretzbacher et al., 2015a).

1.14 Reactor types

Different bioreactor types have been suggested for the AD of various types of substrates. The AD systems can be categorised into two categories based on their mode of operation, with clear differences between them. The first category includes the batch systems (BMPs) while the second includes all the continuous/semi-continuous systems. The difference between these two categories stands on the feeding rate of each system. On a research level, a BMP experiment can be stopped when the daily gas production is less than 1% of the total gas accumulation for three days in a row (Holliger et al., 2016). After the end of the digestion period, the batch reactor must be emptied, cleaned and then loaded again before starting a new cycle. On the other hand, a continuous (or a semi-continuous system) is fed with feedstock on specific rates and all products and by-products either are constantly removed (e.g. continuous system) or at specific periods (e.g. semi-continuous systems). Examples of a continuous system include reactors such as the continuous stirring tank reactors (CSTR) (Risberg et al., 2013), up-flow anaerobic sludge blanket digestion UASBs (Azaizeh and Jadoun, 2010) as well as anaerobic lagoons (Schmidt et al., 2019). Furthermore, another system that has been used to test the anaerobic biodegradability of lignocellulosic residues is the leach bed reactor (LBR) (Cysneiros et al., 2012, 2011; Tian et al., 2017). LBR reactors process feedstock with high solids content without the addition of any stirring in the reactor. Limitations of this type of bioreactors include the usual clogging of the feedstock in the reactor, the low degree of homogeneity and the high operation complexity (Yang et al.,

2020). Finally, a two-stage bioreactor system has been also investigated, in which the processes of hydrolysis and acidification (first two stages of anaerobic digestion) are carried out separately from the acetogenesis and methanogenesis (third and fourth stages of AD respectively) and are held in a different reactor (Cysneiros et al., 2011). The main reason for this approach is that the two sets of processes are not sharing the same optimal conditions (e.g. the hydrolytic microorganisms can adapt to lower pH values compared with the methanogenic archaebacteria) (Wang et al., 2014). Although the two-stage systems are more efficient compared to single-stage systems, the cost for their operation is usually higher (Ward et al., 2008).

1.15 Purpose of WS examination in continuous mode.

Even though the effect of steam explosion (SE) pretreatment on the biomethanation of lignocellulosic residues is well examined, most studies have been conducted in batch mode systems. Although in the literature there is already data that can support the idea that steam explosion pretreatment can significantly increase the outcome of a full-scale biogas plant (Shafiei et al., 2013), there is no extended literature on the use of continuous systems digesting SE straw.

Today, continuous fed bioreactors systems hold the vast majority of the industrial scale bioreactors. Despite the gap in the literature, there is a need for the evaluation of steam explosion pretreatment in continuous or semi-continuous AD systems. Furthermore, there very few studies have examined the microbial population of bioreactors that are continuously or semi-continuously fed with straw. Firstly, several operational and biological parameters of the system, including the organic loading rate (OLR), hydraulic retention time (HRT), accumulation of volatile fatty acids (VFAs) and others, can only be evaluated in a long-term digestion scenario. Secondly, the potential changes

that SE can cause in straw can theoretically result in changes in the anaerobic microbial consortia inside the bioreactor. Also, the biological adaptation of the system to the new steam-exploded pretreated straw can positively contribute to the enhancement of biogas production from lignocellulosic residues, while this parameter can only be tested in a long-term basis.

In one of the few studies that tested the steam explosion treatment in a CSTR reactor, Risberg et al. (2013), used a 5L system operated at three different temperatures (37, 44 and 52 °C) with an OLR of 2.8 gram of VS per litre of reactor per day. Although the co-digestion process between the WS and the cattle manure was relative stable, the pretreatment did not increase the gas yields significantly. Estevez et al. (2014) also used steam-exploded lignocellulosic material (Salix) together with cow manure and tested the recirculation of the liquid digestate. An increase in the gas production was reported, but the system proved unstable in the long-term probably due to the accumulation of solids (Estevez et al., 2014). However, the target of this study was not to examine the effect of the pretreatment but the effect of the recirculation of the liquid fraction of the effluent of the process.

As mentioned above, the use of WS in a CSTR system can create operational problems especially when lab-scale bioreactors are used. Steam explosion pretreatment can significantly reduce the negative effect of this residue on the operation of bioreactors. After pretreatment, the pores of feedstock are opened and as a result, the moisture content increases while the particle size is reduced. Boonterm and his team stated that the fibre surface wettability increased along with the increase in the harness of the steam explosion conditions (Boonterm et al., 2016). Similar results were reported by Bauer et al. (2014) who found out that longer pretreatment's retention times and an increase in the pretreatment's temperature resulted in a lower dry matter content for the steam-

exploded hay samples compared to the untreated ones. As a result, steam-exploded straw holds the potential for reducing the mechanical issues WS usually creates when digested in a continuous AD system which also reduces the risk of unwanted shutdowns of the system.

1.16 Operational parameters during the steam explosion pretreatment

Some of the parameters affecting the efficiency of the steam explosion prior to AD are the temperature, the pressure, the retention time and moisture content in the reactor. It has been shown that the most important parameter is pressure, while moisture content and retention time of steam explosion pretreatment are secondary ones (Aski et al., 2019). The particle size of the material subjected to treatment plays also an important role in the process. Lignocellulosic substrates have been treated with thermal techniques in different temperature and pressure conditions. The already tested pretreatment temperatures range between 125^{o} C (Zhou et al., 2017) and 230^{o} C (Estevez et al., 2012) with contradicting results regarding the optimal conditions for biogas production. Different pretreatment times and temperatures have been previously evaluated for Salix and the best results were obtained for temperatures above 210 ^{0}C (Estevez et al., 2012). Bauer et al. (2014) tested a range of temperatures between 160 ^{0}C and 220 ^{0}C and stated that the highest methane yields from the digestion of hay were produced after pretreatment of 175 ^{0}C for 10 min (Bauer et al., 2014). Similarly, the retention time of the SE pretreatment was also examined by different researchers. Ferreira tested pretreatment retention times between 1 and 15 min and temperatures ranging between 150^{0} C and 220^{0} C for WS and the optimal conditions were 220^{0} C for 1 min (L.C. Ferreira et al., 2013). Although, due to differences in the type of the lignocellulosic feedstock and potential different pretreatment pieces of equipment used

in each one of these studies, a clear estimation for the optimal temperature range cannot be made. With respect to the pretreatment retention time, longer processes have also been tested in the past. More specifically, the steam explosion of WS was evaluated for duration rates between 30 minutes and 120 minutes (Theuretzbacher et al., 2015b) with the highest methane production achieved by pretreatment of 140 °C for 60 min. Different SE equipment in accordance with differences in the operational parameters of each system and also significant changes in characteristics and the chemistry of the WS deriving from different cultivars can partially explain the alterations reported in the literature (Theuretzbacher et al., 2015b).

1.17 Limitations for the steam explosion pretreatment

Hydrothermal pretreatments have many advantages when compared to the other pretreatment technologies, including independence from difficult to manage chemicals since only water is usually used for these processes. With a recirculation unit installed along with the SE reactor, the water can be recovered cleaned and re-used in each pretreatment cycle. This type of pretreatment can offer great potential for the enhancement of the anaerobic biodegradability of lignocellulosic substrates but at the same time, these techniques may result in lower methane yield and longer time required for achieving the maximum biomethane production. The pretreatment conditions must always be evaluated carefully before the process, to avoid creating conditions that can potentially cause inhibition phenomena. In a recent study, Steinbach et al. (2019) worked with variable SE conditions on WS samples and found that at mild conditions (severity factor 4.1) the pretreatment did not offer any improvement, while at moderate conditions (severity factor 4.3) the methane yields were improved. In the same study, when the pretreatment conditions were more severe, methane yields were significantly

decreased. In addition, the insignificant effect of the pretreatment on the biogas production has been also reported (Risberg et al., 2013). The high temperatures used for the pretreatment and the sudden release of the pressure might contribute to the production of phenolic compounds such as furfural or 5-hydroxymethylfurfural (5-HMF). These organic compounds consist of a furan ring and an aldehyde group while HMF also has an alcohol functional group. Furfurals are formed by pentoses (xylose, arabinose) and 5-HMF from hexoses (mannose, fructose, galactose and glucose). Methanogenic activity is inhibited in furfural concentration between 2400 and 3000 mg/L according to Boopathy, (2009). Also, Castro et al. (1994) proved that high temperature (210 °C) during steam treatment resulted in significantly higher losses of sugars in addition to the production of furfurals. Finally in another study, the decrease of xylose after steam explosion pretreatment was again correlated with the formation of furfurals at temperatures close to 210 °C (Horn et al., 2011).

1.18 Aims, objectives and hypothesis

1.18.1 Hypothesis

The present study hypothesised that steam explosion pretreatment can increase the anaerobic biodegradability of lignocellulosic substrates. More specifically:

- It was expected that after treatment, the structure that keeps together the non-anaerobically degradable lignin and the carbohydrates, breaks down and this renders lignocellulosic biomass a more suitable substrate for the AD process. It is also hypothesised that due to the increased availability of easily degradable substrates, the volumetric methane production increases during the AD process.
- It was assumed that a continuous AD system would be able to operate under steady-state for long digestion periods, fed with steam-exploded WS, while

biogas production would remain in higher levels compared to the control bioreactors operated with untreated WS.

- A further improvement on the produced amounts of biogas could be achieved through co-digestion with food waste or other feedstock containing high levels of protein which could balance the low levels of nitrogen in WS.
- The combination of two different pretreatment methods (mechanical and steam explosion) is expected to result in higher biogas production compared to the two pretreatments applied individually.
- Finally, it was hypothesized that the alteration of the AD feedstock from WS to steam-exploded straw would affect the microbial microflora inside the anaerobic bioreactors. Also, after a reasonable period of time, acclimatization to the new feedstock will result in increased biogas production.

1.18.2 Aims

The main aim of this project was to enhance biogas production from lignocellulosic biomass with the use of a pretreatment stage designed to tackle the usually low biodegradability of residues such as WS. The main pretreatment used in this study was a steam explosion pretreatment. Apart from that, another hydrothermal pretreatment (autoclave pretreatment) was also evaluated towards its ability to increase biogas production from WS while a comparison between a steam explosion and a mechanical pretreatment was conducted in order to examine a further increase in the gas production after the combination of the two processes. Finally, an important aspect of this project was the characterization of the consortium of the microorganisms inside the anaerobic bioreactors and the changes that the different feed (treated or untreated WS) can result to the system.

1.18.3 Objectives

A long-term objective of this project was to succeed in a partial replacement of traditional feedstock of AD commercial plants (e.g. energy crops) without any significant reduction in the produced methane yields. The main short-term objective of this PhD project was to enhance biogas production from WS. In order to succeed high methane yields the optimal operation of the bioreactor is required. During this PhD, the optimisation of both batch and continuous anaerobic digestion of untreated and steam-exploded WS was investigated. Apart from the steam explosion pretreatment, the improvement of the biogas production was also investigated by applying additional pretreatment steps including the maceration and/or the fractionation of the feedstock with the main objective to further increase the gas production. Furthermore, co-digestion scenarios of untreated and steam-exploded WS with food waste or inorganic nitrogen were evaluated in order to balance the very high C/N of WS. Apart from the increase in biomethane production, another objective of this project was to examine potential changes in the microbiota inside the CSTR bioreactors for both the untreated and the SE straw over the two continuous experimental periods. Finally, the focus of this project included the minimization of some engineering problems that are rendering the digestion of WS in CSTR systems genuine challenging.

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2 Thermal pretreatment of lignocellulosic biomass as a substrate for anaerobic digestion

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2.1 Abstract

The wide availability and the relatively low cost of lignocellulosic biomass render it a promising material for renewable energy production. Although the digestibility of lignocellulosic materials is usually low, suitably designed pretreatments can significantly enhance the specific volumetric methane production in anaerobic digestion (AD) systems. In the present study, thermal pretreatment of wheat straw (WS) was carried out in an autoclave, operating at three different treatment retention times (30, 60 and 90 min). The biomethanation efficiency was tested in batch scale under four different organic load rates (OLR) [2, 4, 8 and 12 kg volatile solids (VS)/m^3] for a total experimental period of 35 days. For every OLR, the 60 minutes pretreatment seemed to offer higher ultimate methane yields, expressed in m^3 CH_4/kg VS, compared to the rest of the pretreatments. In the cases of OLR 4 and 8 kg VS/m^3, biomass pretreatment resulted in a methane yield improvement by up to 5%. At low OLR (2 kg VS/m^3), methane production improvement reached 105%, while in ORL 12 kg VS/m^3 the methane yields were enhanced by 25%. Finally, for every OLR tested, the addition of the liquid fraction of the pretreatment in the reactors was found to decrease the specific methane production compared to runs that utilised solely the pretreated solid WS residue while the inhibition effect was decreased along with the increase in the OLR. Overall, higher OLR could be favourable in anaerobic digestion systems operating with pretreated lignocellulosic biomass as a sole substrate.

2.2 Introduction

Driven by increasing living standards and expanding the human population, energy demand worldwide is rapidly increasing. Waste production is following the same increasing trend and as consequence the need for a sustainable management strategy is imperative. At the same time, the European Union is actively promoting independence from traditional fossil fuels, such as petroleum, and subsidizes the application of innovative, alternative and sustainable energy sources. In this direction, the UK has set a reduction target in greenhouse gas emissions (GHG) of 80% by 2050 (Spataru et al., 2015). Anaerobic Digestion (AD) is a biological process that can transform the non-utilisable form of energy present in a material, such as cellulose, into renewable fuel, valuable nutrients and other macromolecules. The main product of AD is biogas, which can be used as fuel in boilers and internal combustion engines or can be upgraded and be directly injected into the national natural gas grid. A by-product of the AD process is the digestate which can be used as good quality, non-chemical fertiliser. Even though AD is widely utilised as a valorisation method for low-cost materials, many industrial-scale reactors are still operating with energy crops as AD feed (Barbanti et al., 2014; Naegele et al., 2012). The use of this type of substrates offers substantial biomethane production mostly because it has a high concentration of easily degradable materials, such as sugars. However, the increase in the price of energy crops in accordance with the relatively high energy input required from planting to harvest and storage, drive the need for an alternative feedstock for AD plants. Furthermore, the farming of energy crops is in direct competition with the cultivation of crops intended for human or animal consumption for the available arable land. To avoid this competition, an alternative candidate substrate, with high availability and relatively low cost for AD systems, is lignocellulosic biomass. A typical example of lignocellulosic biomass that can be used as feedstock in AD systems is straw, including wheat straw (WS) (Chandra et al., 2012a). Worldwide, straw is mostly used as feed and animal bedding material, while significant volumes are also utilised as fuel for

district heating. Finally, due to is high availability a small part of the produced biomass is burned or left unused, thus increasing the environmental impact of this material (Chandra et al., 2012a). Despite that the biogas potential of straw is high, its anaerobic biodegradability is usually low due to the rigid structure of cellulose, hemicellulose and lignin (Fernandes et al., 2009). Lignin is a non-anaerobically degradable and non-water soluble amorphous heteropolymer that wraps the sugars and decrease their accessibility for microorganisms in AD (Hendriks and Zeeman, 2009).

In order to improve the digestion efficiency of lignocellulosic biomass, a pretreatment stage that targets fibre breakdown is often necessary to improve solubilisation and biosorption of hemicellulose and, to an extent, the amorphous region of cellulose. Different approaches have been examined by the scientific community in the past, including biological, physical and chemical processes, targeting the disintegration of lignocellulosic building blocks. Examples of such methods are the use of fungi or bacteria (biological pretreatment), acids or bases (chemical pretreatment) and the application of grinders to decrease the particle size of the substrates (mechanical pretreatment). Even though the usage of chemical pretreatments has shown to have a positive impact on the AD process (Mancini et al., 2018), this strategy can potentially cause environmental issues in cases where no additional steps of removing or no neutralization of those chemical compounds are applied. Thermal and high-pressure techniques, such as steam explosion (Zhou et al., 2016) and hydrothermal treatment (Bolado-Rodríguez et al., 2016), outweigh chemical pretreatments mostly due to zero need for use and recovery of chemicals while only water is required for these processes. Furthermore, the operational cost of hydrothermal pretreatments is usually lower compared to the cost of biological pretreatments. As an example, the main drawback of the enzymatic pretreatment on lignocellulosic residues prior to AD is the high cost for the enzymes themselves according to Hosseini Koupaie et al. (2019).

During thermal procedures, biomass structure opens up due to thermal expansion, and this causes

a reduction of particle size and an increase in the pore volume of the substrate. Moreover, the polysaccharides present in the lignocellulosic materials are hydrolysed to simple sugars leading to higher degradation rates from the microorganisms of AD (Zhao et al., 2018). As a result, the efficiency of the whole AD process can be increased while the retention time required for optimum biogas production is often decreased (Theuretzbacher et al., 2015b). Furthermore, Wang et al. (2010) worked with a hydrothermal pretreatment (steam explosion) on lignocellulosic wastes and reported that the lignin structure was disrupted after the application of pretreatment. This disruption can enhance the anaerobic biodegradability of such substrates, while the cellulose content becomes more accessible to the microorganisms of AD and their enzymes (Wang et al., 2010). Even though the hydrothermal pretreatments are well-examined methods towards the increase of biogas production, most studies have focused mainly on the severity factor effect of the pretreatment on the subsequent biomethane potential (Estevez et al., 2012; Menardo et al., 2012). To date, there is no published research dealing with the optimisation of anaerobic digestion of thermally pretreated lignocellulosic substrates.

The main aim of the present study was to establish the effect of different severity factors of WS's thermal pretreatment and their correlation with the OLR of mesophilic anaerobic bioreactors. Parameters such as the pretreatment retention time, OLR and the addition of the liquid generated from the pretreatment process were evaluated and insights on volumetric methane production, hydrolysis coefficient factor and lag phase were provided.

2.3 Materials and methods

2.3.1 Inoculum and substrate

WS which was utilized as a feed substrate for the bioreactors was collected from fields in the wider area of Nottinghamshire, UK. After collection, WS was transferred to the lab in plastic bags. During the next step, WS was manually chopped (2-3 cm) and stored at 20±2°C until further use. The effluent of a full-scale biogas plant, digesting crops and working at mesophilic temperatures (42±2°C), was used as an inoculum source. The inoculum was received in the lab in a 20 l container and it was manually squeezed in order to remove the large undigested particles. Prior to the beginning of the biomethane potential test experiment (BMP), the inoculum was incubated and degassed at 37±2°C for a period of 7 d aiming the minimisation of the endogenic methane production as proposed in previous studies (Zarkadas et al., 2016). For the needs of the second experiment of this study, a new BMP set-up was prepared. In order to avoid digestion inefficiencies related to a non-active inoculum, equal amounts (1 l) of the initial inoculum were mixed with the effluent from the first BMP experiment. The two inocula were daily mixed manually and placed in the incubator for one week, similar to the first experiment's degassing period.

2.3.2 Autoclave Pretreatment of WS

WS was pretreated through a batch mode thermo-mechanical process, by the application of high temperature (140 °C) and pressure (2.75 bar) at three different retention times (30, 60 and 90 min). A bench-scale autoclave working at the above conditions was used as a reactor for the pretreatments. WS samples were placed in 500 mL Scott bottles and deionized water was added in order to obtain a 35% (w/v) solid content and also avoid burning the feedstock as proposed by Rajput et al., (2018). After heat treatment at set conditions, a rapid pressure and temperature drop followed until atmospheric conditions were reached. Solids were separated from the liquid via a filter pump and filter paper (80 um) and subsequently washed with warm water to remove any

water-soluble components remaining on the solids. Finally, the washed solids were placed at 40°C overnight to dry and subsequently were stored at -20 °C until further use.

The severity factor of all the pretreatment was calculated as log (R_0) according to Equation 2-1, used also by Overend and Chornet (1987).

Equation 2-1: $R0 = t * exp(\frac{T-100}{14.75})$

where, t= pretreatment time in min, T=temperature of the pretreatment in °C and 14.75 is the activation energy value under conditions where the process kinetics are first order, following the Arrhenius law as explained by (Iroba et al., 2014).

2.3.3 Preparation of the BMP tests

This study was conducted in two separate stages, while all AD experiments were conducted in a batch mode, using 150 mL anaerobic flasks. In the first stage, four different organic load rates (2, 4, 8 and 12 kg VS/m^3) were evaluated, in combination with WS obtained in different pretreatment durations. In the second stage, the effect of the addition of the liquid fraction (derived from the pretreatment), on the digestion of the thermally pretreated straw samples was examined. All feedstocks, including the untreated WS, the solid pretreated substrate (SPS), and the whole pretreated substrate (WPS) which included the solids and the liquid generated after the pretreatment, blanks and positive controls were examined under the same four OLR (2, 4, 8 and 12 kg VS/m^3). The examined parameters for each of the two experiments are summarised in Tables 2-1 and 2-2 respectively.

The working volume of both experiments was 70 mL, and appropriate quantities of inoculum, substrate and deionized water were added to keep the inoculum to substrate ratio (I/S) constant at a value of 2. This value has been commonly used in the past for BMP tests (Zarkadas et al., 2016). Before each experiment, all vials were flushed with nitrogen gas for 5 min each, to ensure the anaerobic conditions during the digestion. Subsequently, all vials were sealed with rubber

stoppers and aluminium caps and finally incubated at mesophilic conditions (37±2°C) for a total experimental period of 35 days. Methane production was measured on a daily basis, with the application of a liquid displacement meter as described in previous studies (Zarkadas and Pilidis, 2011), while sodium hydroxide was used for scrubbing CO_2 and measuring only CH_4. Blanks trials (vials where only inoculum and water were added) were prepared for all four different OLR and were used to measure the inoculum's endogenous CH_4 production. After the end of each experiment, the obtained values from the relevant blank trials were subtracted from those acquired from the vials with the substrate. Furthermore, for both experiments, vials with microcrystalline cellulose (Avicel) were chosen as positive controls as previously described by (Flores et al., 2015). All experiments took place in triplicate and results of methane production were expressed per unit of volatile solids (g VS) based on the wet biomass, in standard conditions of temperature and pressure.

The biomethane production data of the solid fraction of the pretreated substrate was modelled using a one-phase exponential model (Luna-delRisco et al., 2011). According to the model, the biomethane yield followed Equation 2-2.

Equation 2-2: $Y = Ymax\ (1 - e^{-k(t-t_{lag})})$

Where, Y= biomethane yield at the time (t), Y_{max} = ultimate biomethane yield, k = hydrolysis constant and t_{lag} = lag phase.

Excel solver was used to fitting the model to the biomethane production data.

With respect to the methane production from the second experiment, the model did not provide a good fit for the liquid fraction of the substrate and therefore, in this case, methane potential values were those obtained at the end of the trial (day 35), as opposed to the ultimate methane potential Y_{max}.

Table 2-1: Anaerobic digestion conditions examined in the first BMP experiment

Organic Loading Rate (OLR)	Pretreatment Retention Time
2	0 min
4	30 min
8	60 min
12	90 min

Table 2-2: Anaerobic digestion conditions examined in the second BMP experiment

Organic Loading Rate (OLR)	Feedstock status
2	No Pretreatment
4	60 min pretreatment (only solids)
8	60 min pretreatment (WPS)
12	N/A

2.3.4 Analytical techniques

Total solids (TS) were determined by drying the samples at 105 °C overnight. Subsequently, every sample was ignited at 550 °C for at least 5 h. The VS content was calculated as the difference between the TS content and the produced ash (after the 550 °C drying process) divided by the wet sample weight, in accordance with standard methods for the examination of water and wastewater (APHA, 2005). The nitrogen content of WS was measured with the use of a Kjeldahl Nitrogen (TKN) analyser. Acid soluble lignin (ASL), acid-insoluble lignin (AIL) and the concentration of sugars were measured in the untreated samples, as well as in the solid fraction of the treated WS after a two-step acid hydrolysis process, according to the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al., 2012). Glucose, xylose and arabinose were measured by high-performance liquid chromatography (HPLC) in an Agilent 1260 series system,

coupled with an Aminex HPX-87H column (Biorad) and a DAD and RI detector in series. The temperature of the column was set at 65 °C and a 0.6 mL/min flow rate with 5 mM H_2SO_4 as mobile phase was used while the sample volume was set at 20 μL. Sugars were detected in a RI detector (Agilent) and were quantified based on calibration curves of commercial sugars used as external standards.

The morphological changes caused by the thermal pretreatment were examined with the use of Scanning Electron Microscopy (SEM) (FEI Quanta 600, USA). The SEM used in this study was equipped with a Field Emission Gun (FEG). At least 8 SEM images were taken for both treated and the untreated material in different magnifications and the most representatives are presented in Fig 2-1 (a-d). Prior to the analyses, all samples were dried with the application of a freeze dryer (VirTis SP Scientific sentry 2.0, USA). After that, the dry samples were placed onto aluminium SEM specimen stubs with carbon conductive tabs and then were coated applying a gold coater (Edwards Sputter Coater S150B) in order to increase the conductivity of samples.

2.4 Statistical analysis

All statistical analyses were conducted on Excel software (Microsoft Office 365 ProPlus, version 2013) with a paired student's t-test while the statistical significance was assigned when $P<0.05$.

2.5 Results and discussion

2.5.1 Physicochemical characteristics of treated and untreated WS

The chemical composition of WS before and after the pretreatment was analysed and calculated on a dry mass matter basis. The results from the analytical measurements are presented in Table 2-3. The total solid (TS) content decreased from 50% (w/w) in the untreated WS samples to approximately 18% (w/w) in the thermally pretreated WS. This reduction is equivalent to a ~75% decrease compared to the initial untreated samples, indicating absorption of water by the fibre. Due to the increased temperatures, it is likely that the substrate pores opened and water absorption occurred. In the past, similar results have been reported by other researchers (Theuretzbacher et al., 2015b). Furthermore, Boonterm et al., (2016) found that the fibre surface wettability increases along with the increase in the severity factor of the thermal treatment.

Based on Kjeldahl measurements and a conversion factor of 5.8, which was previously proposed for residues derived from wheat (Fujihart al., 2008), the pretreatment did not affect the crude protein of WS samples while for all the examined feedstock total protein was measured to have values close to 0.5 % W/W. Similarly, slight differences in the crude protein content after a thermal pretreatment has been reported for lignocellulosic residues previously (Bauer et al., 2014). With regards to the carbohydrate content in WS, the cellulose content, expressed as glucose, in the untreated WS samples accounted for 41% (w/w) of the whole biomass. At the same time, the percentage for hemicellulose, expressed as xylose plus arabinose, was 30% (w/w). Finally, the total lignin content, including acid soluble (ASL) and insoluble (AIL) lignin, represented 17% (w/w) of the whole biomass. All these measurements are in agreement with what has been reported for untreated WS in previous studies (Theuretzbacher et al., 2015a). In another study, Ferreira *et al.,* (2014) stated that the percentage of cellulose in raw WS ranged

between 30 and 40%, while the fractions for hemicellulose and lignin, ranged between 20-30% and 10-20% respectively. According to the sugar analysis, the concentrations of cellulose and hemicellulose in the pretreated samples were ranging between 41-44% (w/w) and 31-32% (w/w) respectively (Table 2-3). A similar increase in glucose concentration in the solid product of thermal pretreatment was previously reported (Lizasoain et al., 2016). On the contrary, several studies on lignocellulosic material have shown a reduction of hemicellulose content in the solid fraction after similar thermal treatment (Menardo et al., 2013). The temperature that the pretreatment takes place seems to play an important role in the composition of hemicellulose. Bauer et al. (2009) reported an increase in the hemicellulose content after treatment at 160°C, followed by a hemicellulose content decrease when harsher pretreatments (in terms of temperature) were applied while Garrote et al., (1999) reported that hemicellulose hydrolysis is usually achieved at temperatures between 150 °C and 230°C. Furthermore, a recent study examining the effect of hydrothermal pretreatment on safflower straw showed that the solubilisation of hemicellulose significantly increased when the pretreatment temperature reached 180 °C (severity factor 4.13) (Hashemi et al., 2019). Finally, Theuretzbacher et al., (2015b) reported that the temperature of the pretreatment has a greater effect on the composition of biomass compared to the treatment's retention time.

As a consequence, the relatively low pretreatment temperatures which were used in the present study for the pretreatment of WS (140 °C) can explain the differences with the literature. However, total solubilisation of WS hemicellulose is not necessarily desirable when the under treatment feedstock is to be applied in an AD system, as the hydrolytic microorganisms of AD could degrade hemicellulose-derived oligosaccharides.

Finally, total lignin was the only parameter that was significantly increased in the composition of WS after thermal pretreatment. Even if the ASL seemed to decrease, the AIL lignin content was increased from 16% (W/W) to 20% (w/w) after the 90 min pretreatment. Similar results

regarding the composition of WS after a steam explosion pretreatment have been reported before (Theuretzbacher et al., 2015b) The increase of the lignin content after a hydrothermal pretreatment of lignocellulosic biomass can be attributed to the formation of aromatic compounds, created by reactions of released C5 sugars and their subsequent re-polymerisation to pseudolignin according to Risberg et al., (2013). However, Theuretzbacher *et al.*, (2015) proposed that the amounts of pseudolignin which are formed during the thermal treatment can be anaerobically degradable.

Table 2-3: Physicochemical characteristics of the untreated and the treated WS (solid fraction). Data are expressed as a percentage of dry matter. * Asterisks indicate that the data are expressed in dry matter

Component	Untreated	30 min	60 min	90 min
Severity factor	**N/A**	2.65	2.95	3.13
Total Solids % **(g TS/g wet sample)**	50.00±4.11	17.87±0.61	18.32±0.40	18.82±0.12
Volatile solids % **(g VS/g wet sample)**	47.79±4.16	17.19±0.73	17.61±0.28	17.99±0.03
Crude Protein content* **(g/100g)**	0.51±0.02	0.55±0.06	0.56±0.07	0.55±0.05
Xylose* **(g/100g)**	28.11±0.9	31.41±1.11	29.57±0.84	30.45±0.74
Arabinose* (g/100g)	1.95±0.13	1.9±0.17	1.71±0.84	1.83±0.1
Glucose* **(g/100g)**	41.12±0.49	40.89±2.19	40.89±1.18	43.8±1.42
ASL* **(g/100g)**	1.23±0.2	0.91±0.08	0.94±0.82	0.79±0.08
AIL* **(g/100g)**	16.35±0.23	19.52±0.02	18.85±0.15	20.16±0.02

2.5.2 Scanning Electron Microscopy (SEM) images of WS samples

Apart from possible polymer solubilisation, this work also studied the effect of structure disruption caused by the applied pretreatment on the subsequent biomethane production. Possible alterations on the physical structure of WS related to the pretreatment were evaluated with the use of SEM before and after the application of the different pretreatments (Figure 2-1 A-D). The thermal pretreatment did not highly alter the composition of WS but at the same time, an effect on the structure of this residue was seen. From the pictures, the thermal pretreatment resulted in structural changes with the surface of the steam-exploded samples being rougher and more disordered (Figure 2-1.B- 2-1.D) compared to the untreated samples which appear to be smoother and intact. (Figure 2-1.A). These structural changes can potentially benefit the process of AD by increasing the microbial colonization and attachment on the feedstock (Zhao et al., 2018).

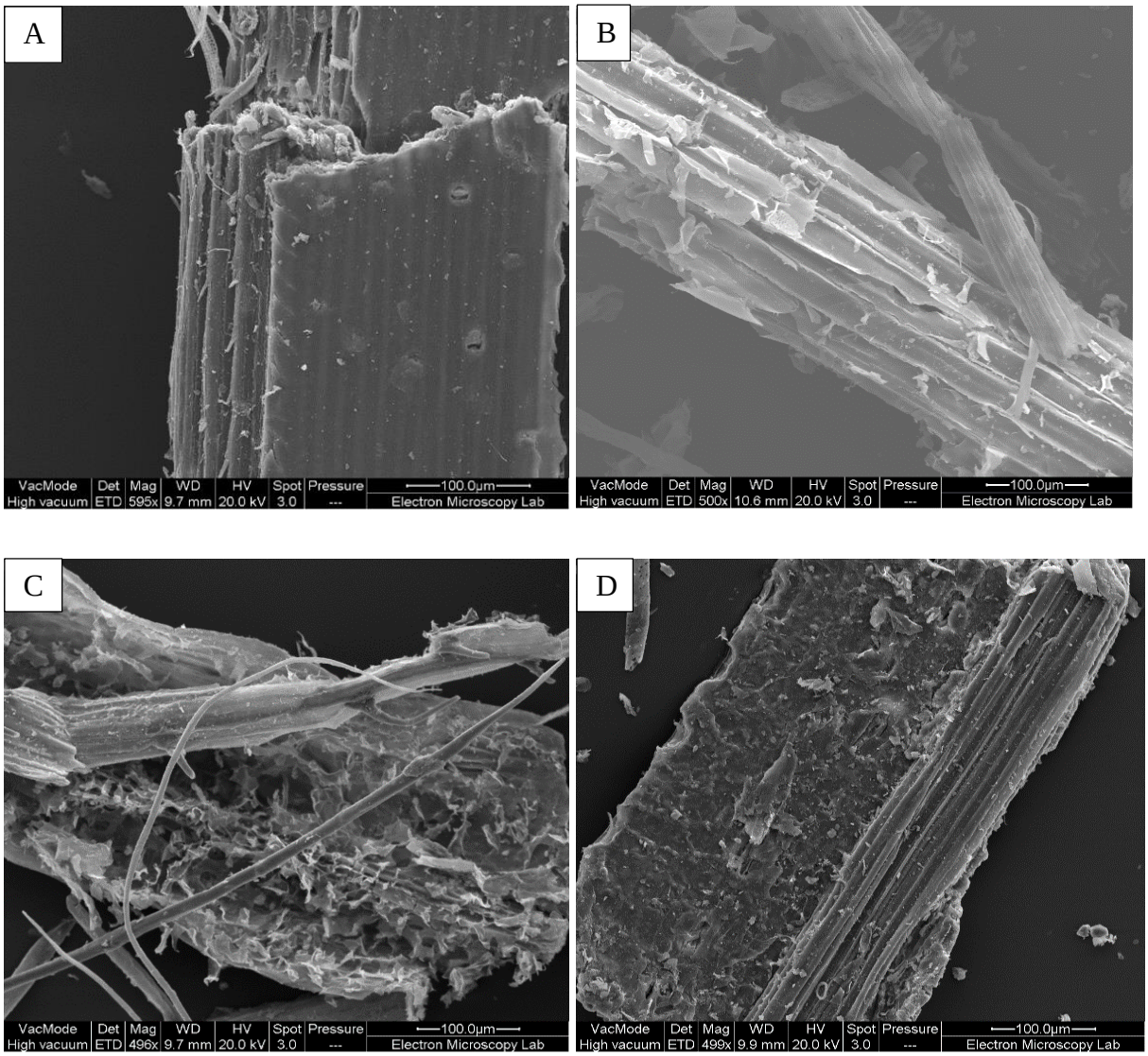

Figure 2-1: 4Scan electron microscope (SEM) images of WS structure of (2-1.a) untreated samples; (2-1.b) 30 min pretreatment; (2-1.c) 60 min pretreatment; (2-1.d) 90 min pretreatment

2.5.3 Specific volumetric CH_4 production

2.5.3.1 First experimental trial

On the first BMP trial of this study, different combinations of the thermal pretreatment retention time and the OLR of the AD system were evaluated. The methane production data were modelled and kinetic coefficients were calculated (Table 2-4), while the predicted methane yields were

also calculated for all conditions tested (Fig. 2-2). The R^2 values indicated a good fit of the experimental data with the model (values >0.95).

Thermal pretreatment, significantly improved the ultimate biogas potential when OLR 2 kg VS/m^3 was applied. Under this OLR, a maximum methane yield of almost 380 m^3 CH_4/kg VS was obtained which was slightly higher compared to results from previous studies (Bauer et al., 2009). Interestingly, the pretreatment did not have the same effect on the ultimate methane potential, which varied between 240 and 280 m^3 CH4/kg VS, when an OLR of 4 kg VS/m^3 was applied. On the other hand, at an OLR of 8 kg VS/m^3, only the 60 min pretreatment time significantly affected the ultimate biomethane potential, which was significantly increased in both cases by ~8%, compared to the gas yields from the untreated substrate. Finally, in the case of the highest tested OLR (12 kg VS/m^3), CH_4 production increased along with the increase in pretreatment time. For this OLR, the methane production for the 60 and 90 min treatments, achieved a yield of 350 and 390 m^3 CH_4/kg VS, respectively (Fig. 2-1 a).

With respect to the lag phase, no significant delays were observed in the AD process for all the different digestion scenarios. According to the modelling data, the longer lag phase was reported for the OLR of 8 kg VS/m^3 with WS solids previously subjected to 60 min pretreatment (1.27 days). The notably short lag phase that was reported in all cases can be attributed to the inoculum source. The composition of energy crops is similar to the composition of WS and this explains the quick adaptation of the anaerobic microflora of the inoculum to the new feedstock. Furthermore, the nature of the substrate used in the present study possible played an important role in the short acclimatisation time required. A faster adaptation of the anaerobic inoculum to substrates rich in carbohydrates, compared to the adaptation to substrates with high protein content, has already been reported (Yang et al., 2015).

The effect of the OLR on the hydrolysis coefficient was clear in the first experiment, indicating the importance of the substrate concentration on microbial growth and activity while at the same

time, the pretreatment conditions had a less pronounced effect on the process. The relationship between the OLR and the hydrolysis coefficient was not linear and followed a parabolic trend, indicating a strong effect of the substrate concentration and availability in the substrate degradation kinetics (Fig. 2-3). The OLR of 2 kg VS/m^3 represents the digestion scenario when the inoculum was diluted the most compared to the rest of the trials. It is possible that this dilution resulted in a decrease of the available microorganisms, especially the methanogenic populations that trends to reproduce slowly according to Ganidi et al. (2009). On the other hand, the decrease in the hydrolysis coefficient in the case of the OLR 12 kg VS/m^3 could be linked with the release on the system of an increased amount of easily degradable components of which resulted in the rapid production of volatile fatty acids (VFAs). This rapid production of VFAs can cause a temporary inhibition to the acetoclastic methanogenesis according to Qu et al. (2009) and as a consequence, this can cause a decrease in methane productivity. Finally, the hydrolysis coefficient trend (Fig. 2-3) suggests that if a full-scale digester is to be operated at low or high OLR, a longer retention time is required compared to a digester operated at OLRs in the range of 4-8 kg VS/m^3.

Table 2-4: Kinetic coefficients calculated the biomethane production data model output

OLR	Conditions	*k*	Y_{max}	t_{lag}	SSR
		(d^{-1}) [1]	(l/gVS) [2]	(d) [3]	[4]
2 kg VS/m³	**untreated**	0.048	0.135	0.056	0.033
	30 min	0.045	0.272	0.966	0.009
	60 min	0.041	0.38	0.308	0.002
	90 min	0.051	0.245	1.078	0.007
4 kg VS/m³	**untreated**	0.060	0.263	0.782	0.002
	30 min	0.063	0.247	0.210	0.006
	60 min	0.066	0.278	0.743	0.009
	90 min	0.063	0.239	0.629	0.006
8 kg VS/m³	**untreated**	0.071	0.243	1.057	0.013
	30 min	0.064	0.291	1.108	0.001
	60 min	0.063	0.283	1.273	0.007
	90 min	0.069	0.268	0.946	0.006
12 kg VS/m³	**untreated**	0.041	0.300	0.73	0.001
	30 min	0.047	0.333	0.545	0.000
	60 min	0.045	0.348	0.518	0.007
	90 min	0.045	0.389	0.966	0.001

[1]Hydrolysis coefficient, [2]Y_{max} volumetric Methane production, [3]Lag phase, [4]Sum of square residuals

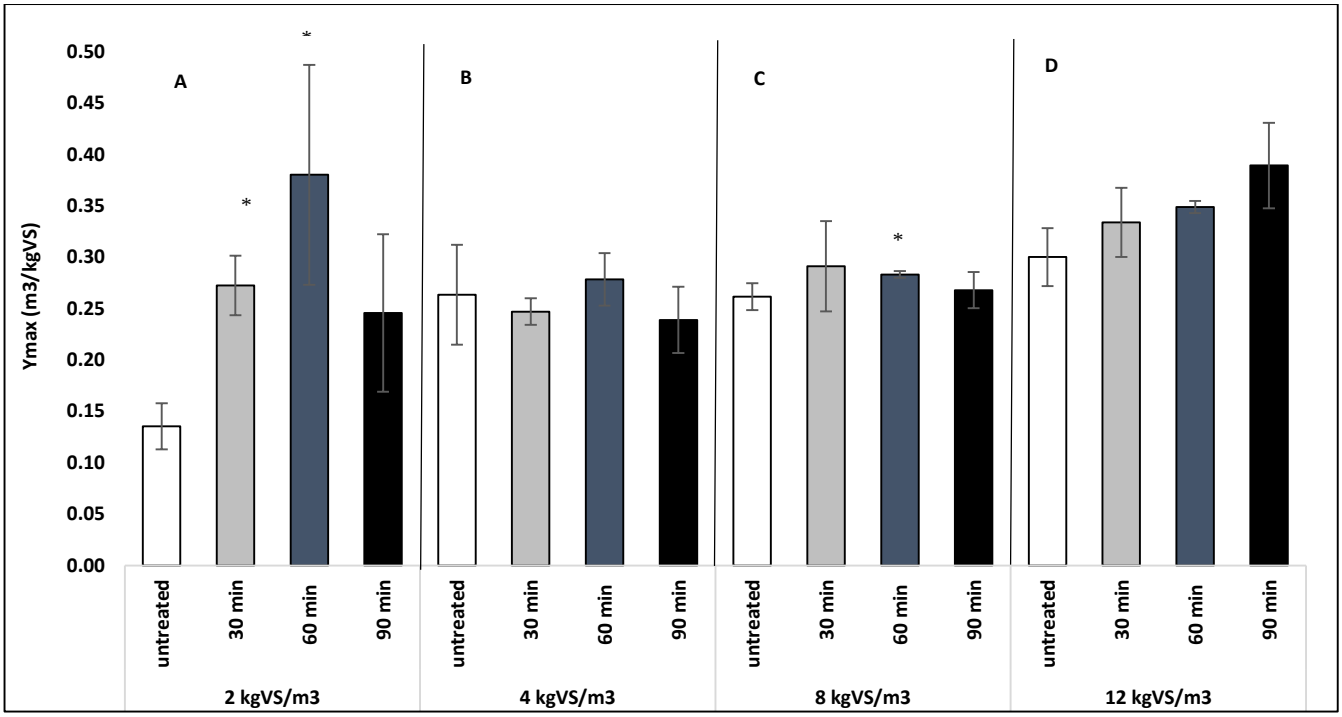

Figure 2-2a: Ultimate volumetric CH_4 yield (Ymax) in batch vial experiments with OLR of (A) 2, (B) 4, (C) 8 and (D) 12 kgVS/m^3, using WS subjected to different pretreatment intensities * Asterisks indicating statistical significance from yields obtained from untreated samples

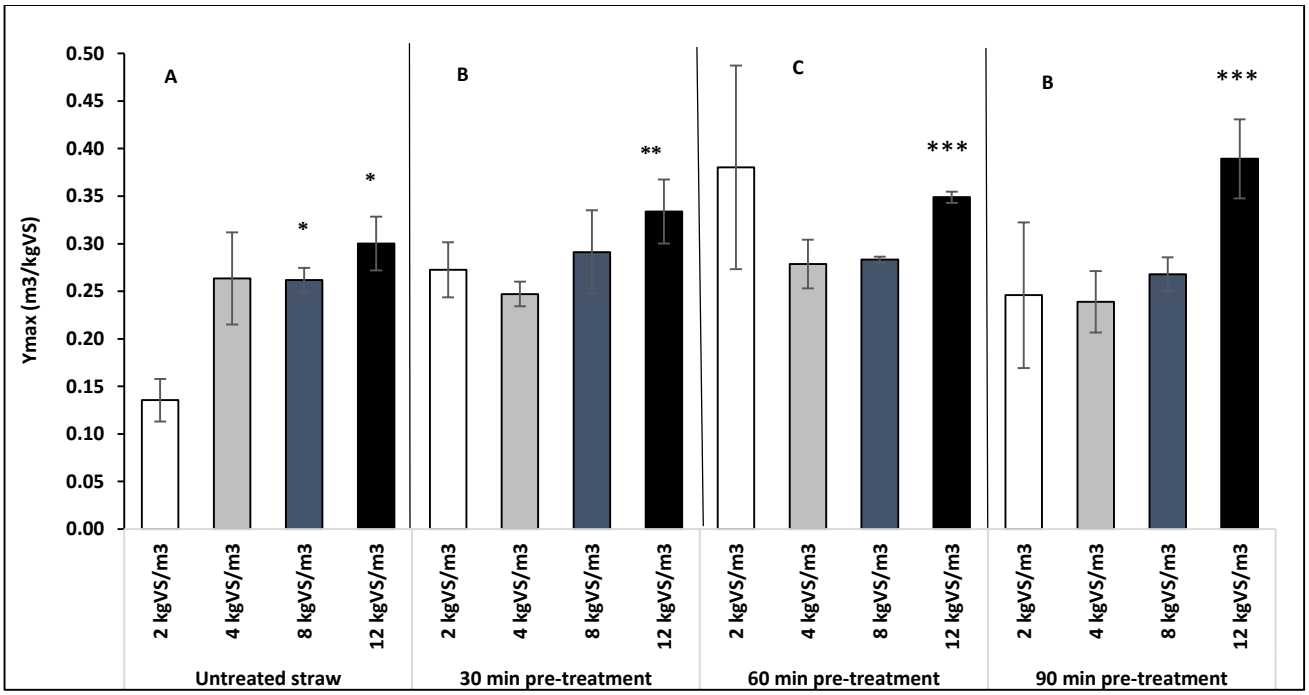

Figure 2-3b: Ultimate volumetric CH_4 yield (Ymax) in batch vial experiments with (A) untreated wheat straw, (B) 30 min pretreated, (C) 60 min pretreated and (D) 90 min pretreatment, at different OLRs (2, 4, 8 and 12 kgVS/m^3) * Asterisks indicating statistical significance from yields obtained from samples with OLR of 2 kgVS/m^3** Two asterisks indicating statistical significance from yields obtained from samples with OLR 4 kgVS/m^3 *** Three Asterisks indicating statistical significance from yields obtained from samples with OLR of 4 and the OLR of 8 kgVS/m^3

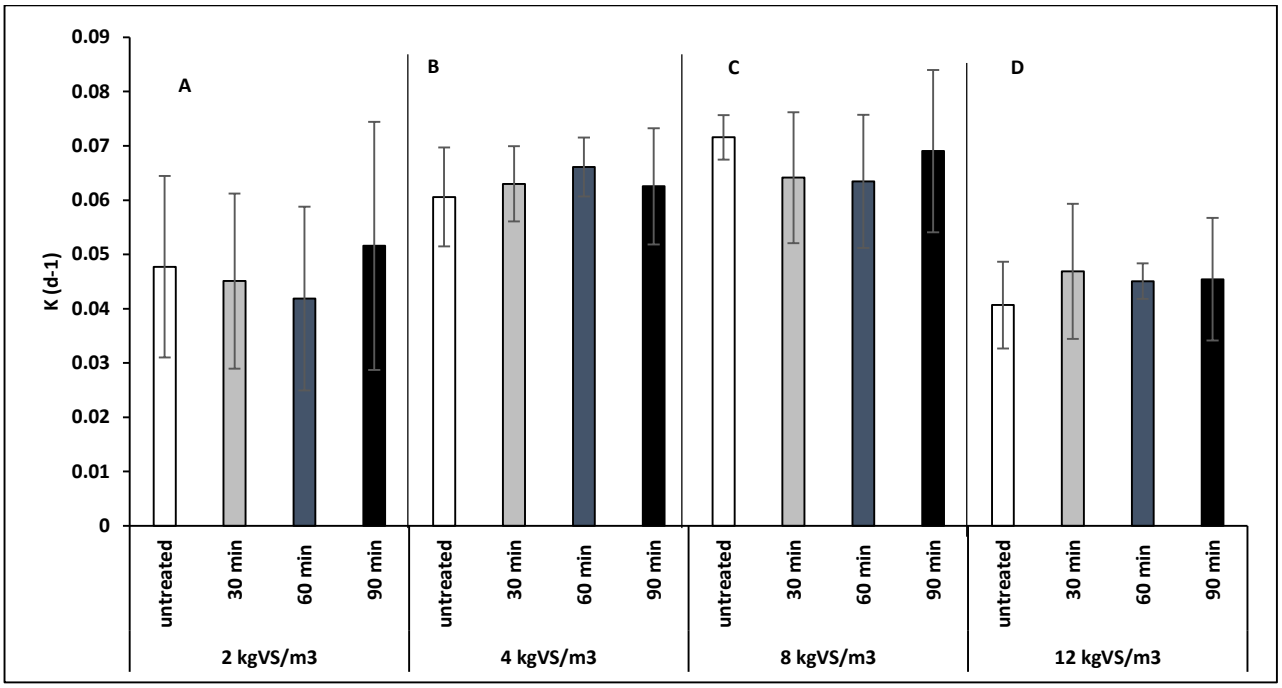

Figure 2-4a*:* Hydrolysis coefficient in batch vial experiments with OLR of (A) 2, (B) 4, (C) 8 and (D) 12 kgVS/m^3, using WS subjected to different pretreatment intensities.

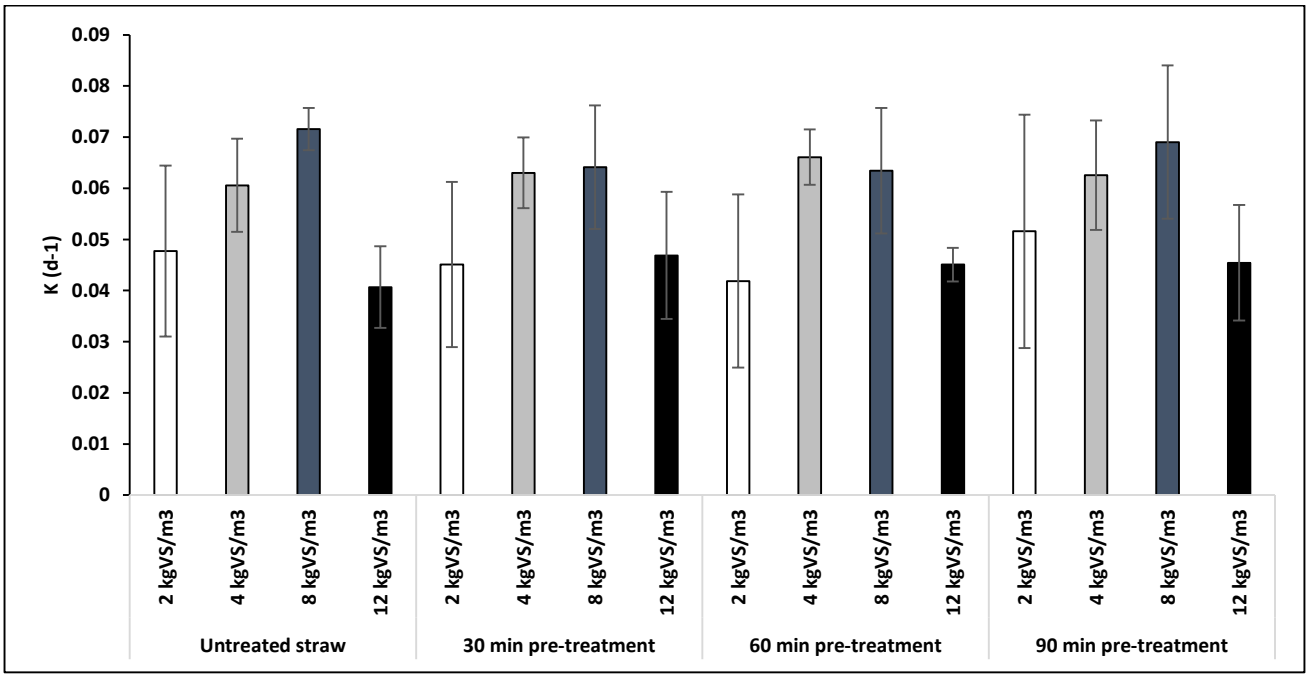

Figure 2-5b*:* Hydrolysis coefficient in batch vial experiments with (A) untreated wheat straw, (B) 30 min pretreated, (C) 60 min pretreated and (D) 90 min pretreatment, at different OLRs (2, 4, 8 and 12 kgVS/m^3).

2.5.3.2 Second experimental trial

In the second stage of the study, the whole pretreated substrate (WPS) from the 60 min pretreatment was tested for its biomethane potential against solely the solid pretreated substrate from the pretreatment (SPS) as well as the untreated (WS) samples which were also used as a control (Fig. 2-4). The 60 min pretreatment was chosen to be tested, due to its higher performance, regarding the produced CH_4 yields, in most of the examined OLR in the previous experimental trial of this study. In all tested OLRs the methane yields obtained from the WPS were lower compared to the yields from the SPS. When the highest dilution of the inoculum was applied (lowest OLR; 2 kg VS/m^3), the CH_4 yield was the lowest for all the substrates tested, indicating that the microorganism availability in the used inoculum played an important role in the digestion efficiency. For this OLR (2 kg VS/m^3), the methane production from the WPS was similar to that of the untreated sample. At the same time, for both OLRs of 2 and 4 kg VS/m^3, methane yields from the WPS were 40% lower than the production from the SPS (Figure. 2-4.a, b), suggesting that the liquid fraction could have contained high amounts of easily degradable material (e.g. weak acids produced after the pretreatment) that caused substrate inhibition to the system or some potential inhibitors (e.g. phenolic compounds or furan derivatives) (Bauer et al., 2014). It has been found in the past that WS contains a significant amount of soluble compounds that can easily be converted into VFAs. Rapid production of VFAs during AD can cause inhibition to the methanogenic archaea of the system according to Rouches et al., (2019) while a possible increase of these compounds in the liquid produced by the pretreatment can potentially explain the reported low biodegradability of WPS. On the contrary to OLRs of 2 and 4 kg VS/m^3, at higher OLRs (8 and 12 kg VS/m^3), no statistically significant differences were observed between the recovered CH_4 yields from the pretreated solid substrate and the samples of the WPS (Figure 2-4.c, d). It is possible that the increase in the initial microbial population helped the system to recover from any inhibition effect caused by the addition of the liquid produced from

the pretreatment.

The modelling equation was also applied to the data of the second stage of the experiment. According to the modelling fit, the R^2 values were higher than 0.95 while it was also observed a decrease in the R^2 values at increasing OLRs of 8 and 12 kg VS/ m^3. However, the model used in the first experiment was not suitable to describe the microbial activity during the second experiment and overestimated the methane production for the two highest OLRs. The estimated Y_{max} value for the OLR of 8 and 12 kg VS/ m^3 was above the theoretical methane potential production for WS according to Kaparaju et al. (2009) who calculated this to be 426 m^3/ kg VS based on the stoichiometric conversion of WS's organic matter to CH_4 and CO_2. A possible explanation for this could be related to the fact that half of the inoculum in the second stage of the experiment was the effluent of the first experiment of this study. Acclimatisation of the inoculum to the pretreated feedstock could have made the microorganisms of the system follow a slightly different kinetic model and as a consequence probably another equation would have had a better fit in this case. Except for the overestimation of the predicted methane yields, it was also clear that while the methane production had stopped for several days, the modelled values continued to increase for OLR 8 and 12 (Fig. 2-5).

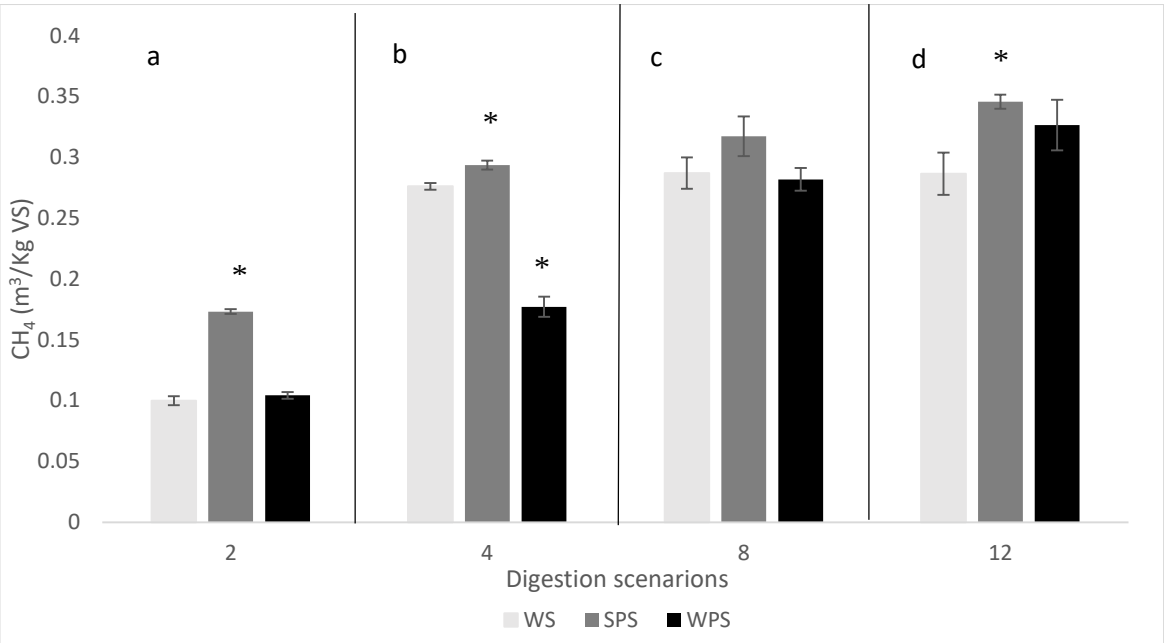

Figure 2-6: Specific volumetric CH_4 production from the digestion of the untreated, the treated solids (60 min treatment) and the WPS with OLR of (a) 2, (b) 4, (c) 8 and (d) 12 kgVS/m^3, after 35 days of incubation * Asterisks indicate statistical significance compared to the yields from the untreated samples ($p \leq 0.05$)

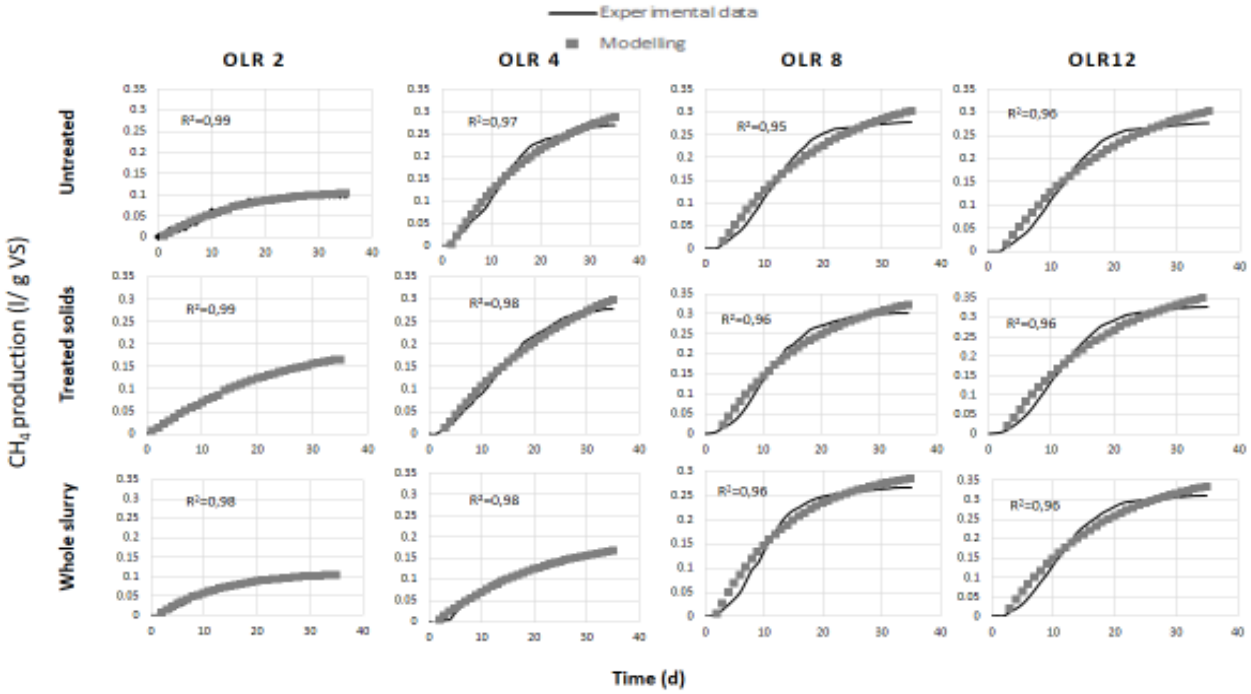

Figure 2-7: Experimental CH_4 production and comparison with the model prediction for the untreated WS, the treated solids and the whole pretreated substrate (WPS) at OLR of 2, 4, 8 and 12 Kg VS/m^3

WS generally represents a promising but, until today, not an ideal substrate for AD systems. The physiology of this residue limits its biodegradability to lower values compared to its potential (Rajput et al., 2018). Its relatively low price, as well as its high theoretical biogas potential, renders it a feedstock worth of investment towards the replacement of traditional AD feedstock, including energy crops. In the present work, the highest predicted biomethane yield from WS obtained from the 90 min pretreatment of WS and the subsequent digestion under the OLR of 12 kg VS/ m^3 (0.425 l/g VS). This amount is even higher compared to the range usually reported for maize (0.375 l/g VS) (Cysneiros et al., 2011) which is one of the most commonly used energy crops with high biomethane potential. This result indicates that using a heat pretreatment may be an efficient strategy towards replacing energy crops with lignocellulosic biomass such as WS. Furthermore, it was found that the AD operational conditions play an important role in the digestion of the thermally pretreated WS and this needs to be taken into consideration when a

similar pretreatment is to be used. Finally, further work is required on the technological side, but also on the feasibility area, since heat pretreatment is energy-intensive and represents an additional cost in the AD process.

2.6 Conclusions

This is the first time that the combined effect of the retention time of a thermal pretreatment (autoclave) and the OLR of an AD system was investigated. The results showed that a thermal pretreatment was successfully applied to WS prior to AD. According to the data obtained, the thermal pretreatment had a more obvious effect on the structure of the biomass rather than its composition, whereas compositionally, only the lignin content on WS seemed to be significantly alternated after the application of the pretreatment. With respect to gas production, the identified optimum conditions were at an OLR of 2 kg/m^3 and 60 min pretreatment and OLR 12 kg/ m^3 with pretreatments of 60 and 90 min. According to these results, batch mode anaerobic bioreactors fed with thermally pretreated lignocellulosic biomass are more likely to handle higher OLRs and/or higher HRT will be required in order to succeed an efficient digestion of this type of feedstock. Furthermore, the addition of the liquid fraction generated after the thermal pretreatment seemed to affect negatively the process. Finally, the results of this study suggest that with further optimisation, higher volumetric methane yields from WS can be achieved, maintaining the biogas output of existing plants and moving a step forward the replacement of energy crops as primary AD feedstock.

Acknowledgements

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3 Enhanced biogas production from steam-exploded wheat straw in BMP and continuous bioreactor systems

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3.1 Abstract

Along with the rapid expansion of the bioenergy sector, there is a need for increasing biogas production from feedstock that they are not coming in direct competition with food production. Wheat straw (WS) is a material with low anaerobic biodegradability, however, after the successful application of a steam explosion pretreatment, WS holds potential in replacing energy crops as a primary feedstock in full-scale biogas plants. In the first stage of the present study, along with measurements on the physicochemical characteristics of untreated and steam-exploded WS, a BMP test was conducted to evaluate the effect of several steam explosion pretreatment conditions on the anaerobic biodegradability of WS. In BMP experiments, thirteen different steam-exploded samples were compared, for their biomethane production, against three untreated WS samples (control). The different pretreatments were conducted under different conditions of temperature and pressure while the severity factor (SF) of the pretreatment was calculated to range between 2.61 and 3.35. The analytical measurements revealed an effect of the pretreatment mostly on the structure of the biomass rather than on its chemical composition. According to the gas production measurements from the BMP trial, the initial biogas potential of raw biomass used for the pretreatment plays an important role in the efficiency of the subsequent digestion. Unfortunately, based on the results from the BMP experiment, no clear conclusions can be made regarding the optimisation of the pretreatment conditions while the highest methane yield was offered from samples that treated at a severity factor (SF) of 2.76, 2.9 and 3.05 respectively. A continuous stirring tank reactor's system was used for comparing the methane production between the untreated wheat straw (WS) and the steam-exploded wheat straw (SE) at two different organic loading rates (OLR) (2 and 5 g VS/L day^{-1}). Steam explosion pretreatment offered increased methane yields on an

average percentage of 20% (ml CH_4/ g VS_{added} day^{-1}) compared to the production from WS for both the examined OLR. Finally, the composition and possible changes in the microbial populations were monitored for both feedstocks over the whole CSTR experiment. Concerning the microbiological analysis, no significant differences were found between the biodiversity inside the reactors digesting SE and WS. However, the increase of the OLR, which also resulted in decreasing the hydraulic retention time (HRT) of the system, seemed to affect to an extent the microbial composition for both substrates. When the OLR was set to 2gVS/l the most dominant order was *Clostridiales* while after the OLR was increased to 5 gVS/l the most abundant order was found to be *Bacteroidales.*

3.2 Introduction

Earth is currently facing an increasing human population which at the same time continuously increases its consumption of food, materials and resources at an alarming rate. Furthermore, several anthropocentric activities are creating problems such as the depletion of its natural resources and rapid climate change. Recently, the research interest in alternative methods to produce the energy required to sustain the increasing population and its needs has risen. One promising technology towards the production of renewable energy and the management of different organic wastes is anaerobic digestion (AD). During this biological procedure, organic materials are catabolised by a consortium of microorganisms, operating under conditions of absence of oxygen, with the main final product being biogas. The main uses of biogas include the production of heat and electricity, while biogas can also be used directly as a fuel for transportation or heavy good (HGV) vehicles.

Over the last decade, energy crops have been commonly used as a primary feedstock not only for lab-scale experiments (Amon et al., 2007; Cysneiros et al., 2011, 2012) but also as feeding material for full-scale AD plants while also representing the main substrate used in the industry in Europe (Naegele et al., 2012). The cultivation of those types of residues for the production of biogas may have disadvantages if not managed well. In some areas, competition for arable land may occur with crops that are intended for human or livestock feed and increases the cost for the production of the latter. Furthermore, the cost for the cultivation of the energy crops and their need for water, especially for countries with low rainfall levels, and fertilizers is escalating the price for the production of biogas. Therefore, the need for finding an alternative as well as cheap feedstock for AD plants is starting to be obligatory. Lignocellulosic residues, such as wheat straw (WS), represent a very promising material for the production of different types of biofuels, including biogas and bioethanol (Theuretzbacher et al., 2015a). The relatively low price of WS, which for the UK was between £10-£15/t in 2020 (Farmers Weekly, 2020), along with its high availability, constitute WS as a promising feeding material for full-scale biogas plants. WS represents the most abundant source of biomass in Europe and the second in the world after rice straw (Talebnia et al., 2010). Currently, large quantities of produced straw are left unused in the fields after harvest, whereas for example in China only half of the produced amounts are used as a bedding material or goes to incinerators in heating plants according to Zhou et al., (2016).

As stated in the previous chapters of this thesis, WS mainly consists of three polymers: lignin, cellulose and hemicellulose. Cellulose is initially much easier to degrade by the microorganisms of AD compared to the other two polymers, while is not readily available to the microorganisms of AD due to the physical barrier that lignin and

hemicellulose structures are creating (Rajput et al., 2018). Lignin is an anaerobically non-degradable polymer (Pohl et al., 2013) and its content in WS is usually higher when compared to other lignocellulosic residues, such as hay (Bauer et al., 2014). At the same time, continuous AD operation on WS is associated with technical challenges relating to mechanical issues which render the use of WS to full-scale biogas plant's reactors challenging. As an example, the floatation of the straw, due to its low density, inside the bioreactor have been reported in the past by Heeg et al., (2014). Furthermore, the possibility of a feedstock accumulation inside the bioreactors, which will increase the reactor's density (Pohl et al., 2013), can also create issues with the mixing of the system. As a consequence, the use of WS as a primary AD feedstock in a continuous bioreactors system is usually problematic. Due to these, a pretreatment stage is usually necessary for breaking down the structure of WS and promote hydrolysis rate and subsequently biogas production. One promising method for the pretreatment of WS prior to AD is a steam explosion (Shafiei et al., 2013). During the first stage of the steam explosion process, the already chopped biomass is placed inside a reactor where high pressure and temperature are used for a short period (Ahmad et al., 2018). The duration of the pretreatment can range between a few minutes to more than two hours (Cui et al., 2012; Theuretzbacher et al., 2015b). During the second stage of the pretreatment, a rapid release of the pressure occurs, usually by opening a valve placed on the reactors vessel, which causes a physical disruption in the structure of the lignocellulosic residues and detaches the lignin from the more degradable under anaerobic conditions components of the biomass.

Various scientific groups have examined the effect of steam explosion pretreatment as a method to improve biogas production from different lignocellulosic residues. Menardo et al. (2013) found out that the addition of a steam explosion step before AD

increased the methane yields produced by Miscanthus (Menardo et al., 2013), while similar improvement was reported by Zhou et al., (2016) on rice straw. However, most of the available data in the literature are based on non-continuous (batch) system experiments (BMPs), while there is a lack of studies conducted in continuous anaerobic reactors systems fed with steam-exploded WS. Taking into account that the vast majority of the existing full-scale biogas plants are operating on a continuous mode, a mimicking of the commercial AD process in a lab-scale CSTR reactor system is necessary to justify the effectiveness of any pretreatment on lignocellulosic biomass. In addition to that, a long digestion period is required to evaluate the effect of steam-exploded straw on several biological factors related to the AD process. These parameters include the microbial population adaptation to the new feedstock and the production of intermediate by-products (VFAs) which can also be considered as potential inhibition factors for the system. In that direction, a BMP experiment was prepared for testing the effectiveness of different steam explosion pretreatment conditions on the anaerobic biodegradability of WS. On the next step of this study, a continuous stirred tank reactor (CSTR) system was used for testing the effect of the pretreatment (160 °C at 6.4 bar) on the continuous digestion of WS for 209 days. Finally, variations on the microbial communities structure and activity in the continuous digestion of both the untreated and the steam-exploded WS were explored using DNA sequencing analyses.

3.3 Material and methods

3.3.1 Feedstock and pretreatment

The straw that was used as feedstock for the bioreactors and the BMPs experiment in the present study was harvested in Norfolk, UK (52.6140° N, 0.8864° E). After

harvesting, the material was transferred to the facilities of Future Biogas Ltd (UK) where it went through a full-scale steam explosion machine (Economizer SE). Before the steam explosion, the lignocellulosic residue was shredded to a size smaller than 5 cm and water was added until a moisture content of 50 % (w/w) was achieved. Finally, just before the pretreatment, the moisture of WS increased to a level of 70% (w/w). In order to succeed in this, the Economizer SE was fed with products and by-products of the subsequent AD process including biogas and water from the digestate recirculation as a method to decrease the environmental impact of the process. In more details, part of the produced biogas was burnt in order to produce the necessary steam for the pretreatment, without the need for extra energy input, while the liquid digestate produced by the AD was recirculated into the pretreatment process in order to avoid further waste of clean water. The Economizer SE operated at a temperature range between 150 °C and 170 °C while the pressure inside the pretreatment vessel was set between 5.8 and 9.5 bar for 14 min. Both samples of untreated wheat straw (WS) and steam-exploded wheat straw (treated at various conditions of temperature and pressure) were transferred in the lab in 20 L plastic containers. Sub-fractions from each material were separated and used for measuring the physicochemical characteristics of both treated and non-treated residues. The remaining amounts of all samples were stored in the freezer at -20 °C until used as feedstock for the bioreactors.

Three different batches of untreated WS arrived in the lab within 9 months. WS_1 arrived at the end of August 2018 while the samples WS_2 and WS_3 arrived in January and March of 2019, respectively. For the SE batch trials, thirteen (13) samples treated under various steam explosion conditions were used. The examined pretreatment conditions lied within a temperature range between 150 °C and 175 °C and the pressure ranged between 5 and 9.5 bar (Table 3-1). The severity factor was calculated based on

Equation 3-1 below, which was adapted from previous studies (Lizasoain et al., 2016) and is also presented in table 3-1.

Equation 3-1: $Log(Ro) = Log(t * exp\left(\frac{T-100}{14.75}\right))$

where t is the pretreatment time (min), T is the pre-treatment temperature (K) and 14.75 represents the activation energy value under conditions where the process kinetics are the first order based on Arrhenius law.

All trials were conducted in triplicate and all the results are presented as mean values along with the reported standard deviations.

Table 3-1: Sample codes from untreated (WS) and steam-exploded (SE) wheat straw used as substrates in BMP experiments, together with treatment conditions and severity factor

Sample	Temperature (°C)	Pressure (Bar)	Severity factor
WS_1 (August 2018)	N/A	N/A	N/A
WS_2 (January 2019)	N/A	N/A	N/A
WS_3 (March 2019)	N/A	N/A	N/A
WS	N/A	N/A	N/A
SE	160	6.4	2.9
SE 02	160	5.8	2.9
SE 03	160	6.4	2.9
SE 04	160	6.4	2.9
SE 05	165	6.4	3.05
SE 06	165	7.4	3.05
SE 10	170	7.4	3.2
SE 12	175	8.4	3.35
SE 13	175	9.5	3.35
SE 14	163	9.5	3
SE 15	163	7	3
SE 16	163	7	3
SE 17	155	7	2.76
SE 18	150	5.8	2.61

3.3.2 BMP experiment

Batch mode assays were assessed to investigate the biomethane potential (BMP) of different untreated WS samples as well as steam-exploded straw samples, using the protocol previously described by Zarkadas et al. (2019). The BMP experiment was conducted in glass vials with a total volume of 150 mL and the working volume of 70 mL. The effluent of a full-scale biogas plant, digesting crops and working under steady-state, was used as an inoculum source for the BMP experiment. Prior to the beginning of the experiment, the inoculum was sieved (2 mm) for removing the undigested big particles and then incubated for seven days at 42 °C as a method to minimise the endogenous biogas production. BMP vials with cellulose microcrystalline (Avicel® PH-101, Sigma Aldrich) were used as a positive control as described earlier (Tsapekos et al., 2017a). Blank vials, containing only the anaerobic inoculum, were used to calculate and subtract from the BMP results the methane production coming from the inoculum. With regards to the preparation of the BMP experiment, after the addition of the inoculum, water and feedstock, all vials were flushed for 5 min with N_2 and subsequently sealed with aluminium caps, aiming to create the necessary anaerobic conditions. The organic load rate for all the trials was set at 6 g VS/L while the inoculum to substrate ratio was (I/S) 3.7 as has already been identified in the past as appropriate for BPM tests on lignocellulosic residues (Eskicioglu and Ghorbani, 2011). The homogenisation of the BMPS was conducted every 12 h by manually shaking each vial for 2 min.

3.3.3 Operation and configuration of the CSTR bioreactors

The effect of steam explosion pretreatment on the anaerobic biodegradability of WS was tested on a four bench-scale CSTR bioreactor system for a total period of 209 days. All four bioreactors were operating at mesophilic conditions (42 °C), maintained through a continuously heated water jacket. The temperature inside all bioreactors was manually measured once per day with an external thermometer. Two of the bioreactors were daily fed with untreated WS, while the second pair of the reactors were fed with SE. The total volume of the reactors was 5 L while the working volume was set at 4 L. All reactors were fed daily at a specific time from a small sampling port on their top and subsequently, those holes were sealed with a rubber stopper. The effluent was removed occasionally to maintain the working volume stable at the desired level. After collection, the effluent of the reactors was stored in plastic jars placed inside an incubator working on mesophilic conditions (42 °C) in case a re-inoculation of the reactors was needed. The four plastic jars were also manually shaken every day to avoid the flotation of suspended particles. Each reactor was equipped with an electric motor (Mellor Electric DC Geared Motor, 24 V dc, 3.9 Nm, 80 rpm) which was connected and provided energy to a stirrer containing plastic paddles. As inoculum source for the first continuous digestion trial, it was used the effluent of a full-scale biogas plant digesting energy crops at mesophilic conditions (42 °C). Due to the mechanical issues that occurred in the early days of the experiment, feeding was suspended for 7 days and recommenced after that. This was considered day 0 of the trial. Subsequently, the untreated WS was manually chopped with the application of scissors on a particle size ≤ 3 cm. Due to the same mechanical issues that WS, especially the non-steam exploded, caused on the mixing system, it was also decided the replacement of the agitation paddles. The new paddles were made of stainless steel to avoid corrosions caused by

the water inside the bioreactor while their size was decreased at a level of 1 cm in each direction to succeed in better mixing of the reactors. After the replacement of the stirrers, the problems with the mixing of the reactors were minimized. The necessary energy for the mixing of the reactors was provided by two power supplies (Manson EP-925 Manson 25A 3-15V Power).

Regarding the operational conditions of the system, the OLR of the reactors was set at a level of 2 g VS/L and subsequently was increased to 5 gVS/L after a working period of 107 days. It is also worth mentioning that due to differences in the moisture content between WS and SE, an additional amount of water was decided to be added daily in the two digesters fed with WS in order to balance the hydraulic retention time (HRT) between the two systems. For both feedstocks, the HRT during the first experimental period (OLR of 2kg VS/L) was calculated to be almost 100 d while decreased to 40 d after the increase in the feeding rate of the system. The reactors' performance was continuously evaluated by measuring daily biogas production using the water displacement method as described before by González-fernández et al., (2013). Apart from the methane production, other important parameters were used as indicators for monitoring the operation of the system including the pH, the volatile fatty acids (VFA) production and the degradation and accumulation of volatile solids (VS) inside the bioreactors. All the above parameters (excluding the gas production) were collected once every seven days.

3.3.4 Analytical methods

Total solids (TS) of untreated WS and SE, the inoculum and the effluent of both the bioreactors and the BMPs were determined by weighing samples in porcelain crucibles and subsequently drying them at 105°C (Gallenkamp (UK)) overnight. Every crucible

along with the dried sample was ignited at 550°C (Carbolite, England) for at least five hours. The volatile solids (VS) content was calculated as the difference between the TS content and the produced ash (after igniting at 550°C) divided by the wet sample, under the standard methods for the examination of water and wastewater (APHA., 2005). The composition of the different AD feedstock in carbohydrates and acid-soluble and insoluble lignin (ADL and AIL) was measured as explained in the protocol of the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2012). Prior to this, briefly dried samples were passed through a coffee grinder (Delonghi, USA) to decrease their particle size lower than 0.05 mm. Triplicates of all samples were mixed with deionized water, at a temperature of 40-60°C to create a solution containing 5% solids (w/w). Samples were placed in a water bath operating at 40°C along with agitation at 80 rpm for 40 min passed through an 11 μm Watman filter paper to separate the water-soluble from the non-soluble solid parts of straw. HPLC was used to examine the concentration of the water-soluble components in samples. Subsequently, the filtered solid fraction was collected and used in the protocol of the two steps acid hydrolysis described earlier by Sluiter et al., (2012).

The concentration of volatile fatty acids (VFAs) in the effluents of the reactors was also measured. Bioreactor effluents were collected and centrifuged at 8500 rpm and a temperature of 10 °C for 20 min, followed by acidification of liquid samples by the addition of formic acid (98% (v/v) concentration) on a ratio of 1 μL per 1 mL of aliquot. Subsequently, the samples were centrifuged again at 8500 rpm and a temperature of 10 °C for 15 min. Finally, the remaining aliquots were filtered through a syringe filter (0.22 nm) and analysed by HPLC.

The concentration of sugars volatile fatty acids, furfural and 5-HMF was measured by High-Performance Liquid Chromatography (HPLC) in an Agilent 1260 series system,

coupled with an Aminex HPX-87H column (Biorad) and a DAD and RI detector connected in series. The temperature of the column was set at 65 °C, with a 0.6 mL/min flow rate (5 mM H_2SO_4 as mobile phase) and a sample volume of 20 μL per injection. Detected compounds were identified based on their elution time and were quantified with calibration curves of external standards (Fisher Scientific, USA).

Measurements of the temperature and the pH inside the bioreactors were conducted at least once every seven days with the use of a thermometer (Fisherbrand™ Liquid Filled Partial Immersion Thermometer) and a pH meter equipped with a microelectrode (Mettler Toledo™ FiveEasy™ Plus FP20 pH/mV Meters) respectively.

3.3.5 Scanning Electron Microscopy (SEM)

Changes caused by the pretreatment on the morphology and the structure of the straw samples were monitored by Scanning Electron Microscopy (SEM) on FEI Quanta 600 SEM equipped with a Field Emission Gun (FEG). Samples (one from each feedstock) were firstly freeze-dried (VirTis SP Scientific sentry 2.0, USA) for 5 days, placed onto aluminium SEM specimen stubs with carbon conductive tabs and covered by a gold coater (Edwards Sputter Coater S150B). Two different magnifications were chosen to be presented (500 x magnification and 6000 x magnification) in order to show the effect of the pretreatment on the structure of WS.

3.3.6 Fourier Transform Infrared Spectroscopy (FTIR)

The effect of the steam explosion pretreatment on the functional groups of WS was also evaluated with the use of Fourier transform infrared spectroscopy (FTIR). The equipment used in this study was a Perkin-Elmer Spectrum 100 FTIR (UK) equipped

with a universal attenuated total reflectance (ATR) scanning accessory. The spectra area ranged from 4000 to 650/cm after 25 times scanning on a resolution of 4 cm^{-1}. For the preparation of the samples, 10 gram of each one of the two frozen bioreactor's feedstocks stocks (WS and SE) were isolated and subsequently left to defrost and oven-dry for 24 hours at 70 °C. During the next step, samples were ground to decrease their size up to a powder level (particle size ≤ 0.05 mm) and were subsequently dried in a freeze dryer for five days, prior to FTIR analysis.

3.3.7 X-ray diffraction (XRD)

The crystallinity of WS samples before and after the steam explosion pretreatment was determined by X-ray diffraction (XRD) as previously explained (Yao et al., 2018). For these measurements, the two bioreactor's feedstocks (WS and SE) along with all the treated samples from the BMP experiment were chosen to be examined. The XRD measurements data were collected on a Bruker D8 advance powder diffractometer and the radiation used was copper K alpha radiation. The voltage was set at 40 kV while the current was set at 40 mA. The data were used in reflection on a flat plate and collected by a Lynxeye detector which operated from 5 to 65 degrees 2 thetas as a raw file and analysed using Bruker EVA software.

3.3.8 Microbiological analysis

Reactor's effluent samples were processed according to the instructions of the isolation kit QIAamp® RowerFecal® DNA Kit (QIAGEN, Germany) protocol for the isolation of DNA from gut material, stool samples and biosolids. The concentration of DNA on the isolated samples was quantified with the use of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Rockland, DE). After that, all samples

were exported to an external laboratory for running the microbial amplicon-based metagenomics sequencing (Illumina PE250, Q30 ≥75%). Total genome DNA from samples was extracted using the CTAB/SDS method. The DNA concentration and purity were monitored on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL using sterile water. The Amplicon Generation was conducted using 16S rRNA/18SrRNA/ITS genes of distinct regions (16S V4/16S V3/16S V3-V4/16S V4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified used specific forward primer 341F (CCTAYGGGRBGCASCAG) and a reverse 806R (GGACTACNNGGGTATCTAAT). For the Archaeal 16S DNA the hypervariable regions V4-V5 (397bp) were used using the primers Arch519F (CAGCCGCCGCGGTAA) and Arch915R (GTGCTCCCCCGCCAATTCCT) were processed. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Next, a mixture of equal volumes of 1X loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% (v/v) agarose gel for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further experiments. Subsequently, PCR products were mixed in an equivalent ratio and then, the mixture of the PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEBNext® Ultra DNA Library Prep Kit for Illumina, following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina platform and 250 bp paired-end reads were generated. All microbial community members found in a concentration below 1% of the total population were disregarded as proposed by Strang et al. (2017)

3.4 Statistical analysis

All standard deviations reported in the present study were calculated using the Excel software (Microsoft Office 365 ProPlus, version 1908). The statistical analyses were conducted with a paired student's t-test and statistical significance was assigned to $p<0.05$.

3.5 Results and discussion

3.5.1 Physicochemical characteristics

Analytical measurements conducted on the different untreated and steam-exploded straw samples revealed that the pH of the feedstock was one of the physicochemical characteristics of WS that were significantly changed after the pretreatment. More specifically, for the WS samples, the pH was measured to be close to the neutral area (pH 6.9 to 7.3) while within the different SE samples the pH was measured closer to the acidic area (pH ranges from 5.2 to 6). A similar decrease in the pH of WS was previously reported by Han et al. (2010) who also measured a reduced buffer capacity for WS after the steam explosion pretreatment. The explanation for this result might be connected with the formation of some weak acids (acetic acid and formic acid) during the steam explosion process (Cubas-Cano et al., 2020). As seen in table 3-1 for the untreated straw feedstock used in the BMP experiment (WS_1, WS_2, WS_3), no significant differences were reported regarding the hemicellulose content. On the other hand, the cellulose (glucose) was found to significantly differ within the samples while measured to range between 36.63 and 46.47 % (w/w) for the three different samples. Similar glucose content [46.86% (w/w)] was measured for the untreated straw used as a bioreactors feedstock (WS). For the same feedstock (WS) the hemicellulose content was measured to represent almost 25% of the total biomass. Regarding the effect of the

pretreatment on the composition of straw, SE did not significantly differ from WS while it was found to have a cellulose content of 47% and hemicellulose content of 28% (w/w). For the BMP pretreated feedstocks, the cellulose and hemicellulose content was within the range of 39-50% and 18-26% of the total biomass respectively. Finally, lignin was found to represent a percentage of 9.53 % (w/w) in WS_1 while for WS_2 and WS_3 lignin found to be significanlty lower (7.6%) for both samples. After the application of the steam explosion the lignin content was not significantly changed while holding values between 9 and 10.5 % (w/w) of the total biomass. Similarly, lignin content was also not significantly affected by the pretreatment in the case of the CSTR feedstock (WS and SE).

Next, in order to examine potential alternations on the structure of WS after the application of the steam explosion pretreatment, pictures are taken by an SEM instrument, of the two bioreactor's feedstock (WS and SE) were compared against two different magnifications. As illustrated in Fig. 3-1, the structure of the lignocellulosic biomass was partially deconstructed after the steam explosion. More specifically, the structure of WS as shown in Fig 3-1A and 3-1C (on 6000x and 500x magnification respectively) was smooth and intact. On the contrary, for SE as seen in pictures 1B and 1D, the structure seems to be more disrupted. A similar effect was reported in previous studies for steam explosion or other hydrothermal pretreatments before the AD of lignocellulosic residues (Li et al., 2016; Zhou et al., 2017).

Table 3-2: Composition in lignin and carbohydrates for the different feedstock used in the present study. All values are expressed on a dry matter basis (w/w)

	Glucose	Xylose	Arabinose	Total Lignin	Mass balance (carbohydrates and lignin)
WS_1	41.90 ± 3.35	28.49 ± 0.34	0.88 ± 0.24	9.53 ± 0.22	80.80 ± 4.16
WS_2	46.47 ± 1.93	28.26 ± 0.61	1.83 ± 0.03	7.62 ± 0.13	84.18 ± 2.71
WS_3	36.63 ± 0.21	27.43 ± 0.6	0.61 ± 0.21	7.78 ± 0.82	72.44 ± 1.85
WS	46.86 ± 0.14	23.47 ± 0.18	1.93 ± 0.39	8.60 ± 0.3	80.87 ± 1
SE	47.54 ± 0.96	27.39 ± 0.08	0.13 ± 0.12	10.07 ± 1.32	85.13 ± 2.51
SE_{02}	41.79 ± 2.03	25.78 ± 2.37	0.45 ± 0.61	10.53 ± 0.01	78.55 ± 5.03
SE_{03}	41.40 ± 4.88	27.80 ± 0.78	0.23 ± 0.02	8.61 ± 0.53	78.05 ± 6.25
SE_{04}	42.81 ± 3.48	28.30 ± 1.21	1.33 ± 0.14	9.45 ± 0.09	81.89 ± 4.94
SE_{05}	45.45 ± 1.11	29.73 ± 0.69	1.52 ± 0.34	9.25 ± 0.23	85.94 ± 2.38
SE_{06}	47.13 ± 0.81	25.01 ± 0.25	1.78 ± 0.48	8.94 ± 0.88	82.86 ± 2.42
SE_{10}	50.61 ± 1.25	17.96 ± 0.36	0.27 ± 0.07	10.18 ± 0.18	79.01 ± 1.87
SE_{12}	41.92 ± 1.28	27.81 ± 0.67	0.33 ± 0.08	9.71 ± 0.7	79.78 ± 2.74
SE_{13}	42.23 ± 6.25	21.31 ± 3.65	0.12 ± 0.05	10.98 ± 0.08	74.64 ± 10.04
SE_{14}	41.85 ± 5.51	22.48 ± 1.94	0.23 ± 0.05	10.93 ± 0.27	75.49 ± 7.78
SE_{15}	46.31 ± 1.99	24.40 ± 0.35	0.95 ± 0.88	10.70 ± 0.49	82.36 ± 4.16
SE_{16}	43.98 ± 4.82	20.13 ± 1.65	1.61 ± 0.46	9.58 ± 0.21	75.31 ± 7.15
SE_{17}	45.91 ± 2.55	22.09 ± 1.27	1.67 ± 0.24	10.13 ± 0.32	79.81 ± 4.39
SE_{18}	39.64 ± 3.35	20.26 ± 1.04	1.36 ± 0.33	9.85 ± 0.63	71.10 ± 5.36

According to the data derived by the analytical measurements and presented in table 3-2, WS samples deriving from the same geographical area, but harvested in different periods of the year (WS_1, WS_2 and WS_3), found to have differences mostly in their cellulose rather than hemicellulose composition. In more details, their cellulose content was measured within a range of 36% to 47% (w/w) while their hemicellulose content, calculated as the sum of xylose and arabinose content in each sample, was found to be between 28% and 30%. Furthermore, steam explosion pretreatment did not seem to dramatically affect the composition in sugars and lignin of neither WS_1 which was used as a starting material for preparing the different pretreated samples (SE_{02}-SE_{18}), nor WS which was the initial material for the steam explosion pretreatment (Table 4-2). Several studies have already stated that the solubilisation of hemicellulose can usually be succeded at temperatures between 150 °C and 230 °C (Garrote et al., 1999) and as a consequence, the relatively low temperatures used in the present study can explain the insignificant change of the hemicellulose content. Furthermore, in previous studies examining the effect of a steam explosion pretreatment prior to the AD of lignocellulosic residues, a decrease in the presence of hemicellulose was also associated with the increase of the SF of the pretreatment (Lizasoain et al., 2016; Theuretzbacher et al., 2015b). In both the above studies, the presence of hemicellulose was not highly reduced after pretreatments with SF up to 3.00 while it was found to almost disappear when SF increased to values close to 4. It is possible that a further increase in the SF of the steam explosion pretreatment used in the present study would have resulted in total solubilisation of hemicellulose which however would not be preferable since some hemicellulose-derived oligosaccharides can be anaerobically degraded to biogas as explained in chapter 2 of this thesis.

3.5.2 FTIR analysis

FTIR analysis was performed for the two bioreactors feedstock (WS and SE) while the results from these are presented in Figure 3-2. The main aim of this analysis was to determine the WS's organic matter transformation after the application of the steam explosion pretreatment (SF 2.9). According to Figure 3-2, the two spectra for the untreated and the steam-exploded straw were almost identical. Similar peaks to the ones found in the present study for both two spectra have also been reported for untreated WS in a previous study examining the effect of liquid digestate pretreatment on the AD of WS (Liu et al., 2019). As an example, results reported by Liu et al., (2019) included two peaks at 3400 and 2920 cm^{-1} attributed to the O-H stretching and C-H stretching vibration respectively, while the same peaks have also been found in the present study for both WS and SE. The highest peak areas were observed both for WS and SE at 1030 cm^{-1} which is quite similar to the peak at 1064 cm^{-1} previously connected to cellulose and hemicellulose in rice straw (Aski et al., 2019). However, SE also found to present an additional small peak, compared to WS, at 1634 cm^{-1} which is very close to the values reported to represent the C-O bonds in the alkyl groups (1640 cm^{-1}). According to Gu et al., (2015), the C-O bonds can be found on the lignin side chains (Gu et al., 2015). The observation of this extra peak in SE samples can validate the results presented in table 3-1, indicating an increase in the lignin content after the application of the steam explosion pretreatment on WS.

3.5.3 X-ray diffraction analysis

X-ray diffraction analysis on both untreated (WS) and steam-exploded straw (including SE and the pretreated feedstocks used in the BMP experiment) were conducted for identifying potential alternations on the crystallinity index (CI) after the use of different pretreatments. According to the results from these measurements, there were reported

insignificant changes in the crystallinity index of the biomass after the use of the pretreatment Fig (3). In more details, the CI in the untreated straw samples was close to 74% of the total biomass, compared to 75% in all steam-exploded samples. These results seem not to be in agreement with previously published reports where an increase in the crystallinity factor on rice straw samples was found after a steam explosion pretreatment (Aski et al., 2019). In another study, WS passed through a thermal pretreatment at four different temperatures (120, 140, 160 and 180 °C) while the increase in the pretreatment temperature seemed to increase the crystallinity index (Rajput et al., 2018). The increase in these studies was attributed to the solubilisation of hemicellulose and lignin, which mostly constitutes the amorphous region of the biomass, after the pretreatment. In the present study, the water-soluble parts of WS were found to represent a very small percentage of the total biomass (results are not presented here) and as a result, the insignificant alternation of the crystallinity can be explained. In addition, un-similarities on the pretreatment method as well as variations on the composition of the AD feedstock can also explain this difference.

As a consequence of the above measurements, the effect of the steam explosion pretreatment was mostly clear on the structure of the biomass as seen in SEM pictures (Fig 3-1 A to D) rather than the chemical composition of it (Fig. 3-2), (Fig.3-3) and (Table 3-2).

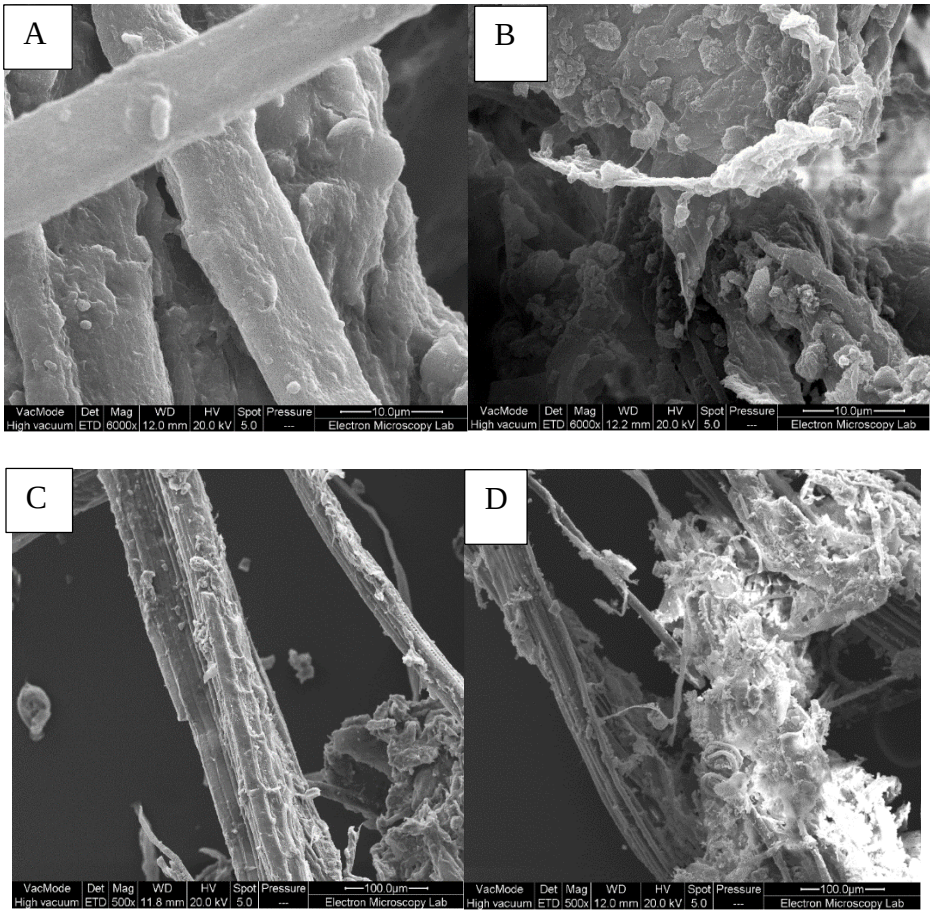


Figure 3-1: Scan electron microscope images from the untreated (A), (C) and the steam-exploded at a temperature of 160 °C and pressure of 6.4 bar (B), (D) on two different magnifications (6000x and 500x respectively)

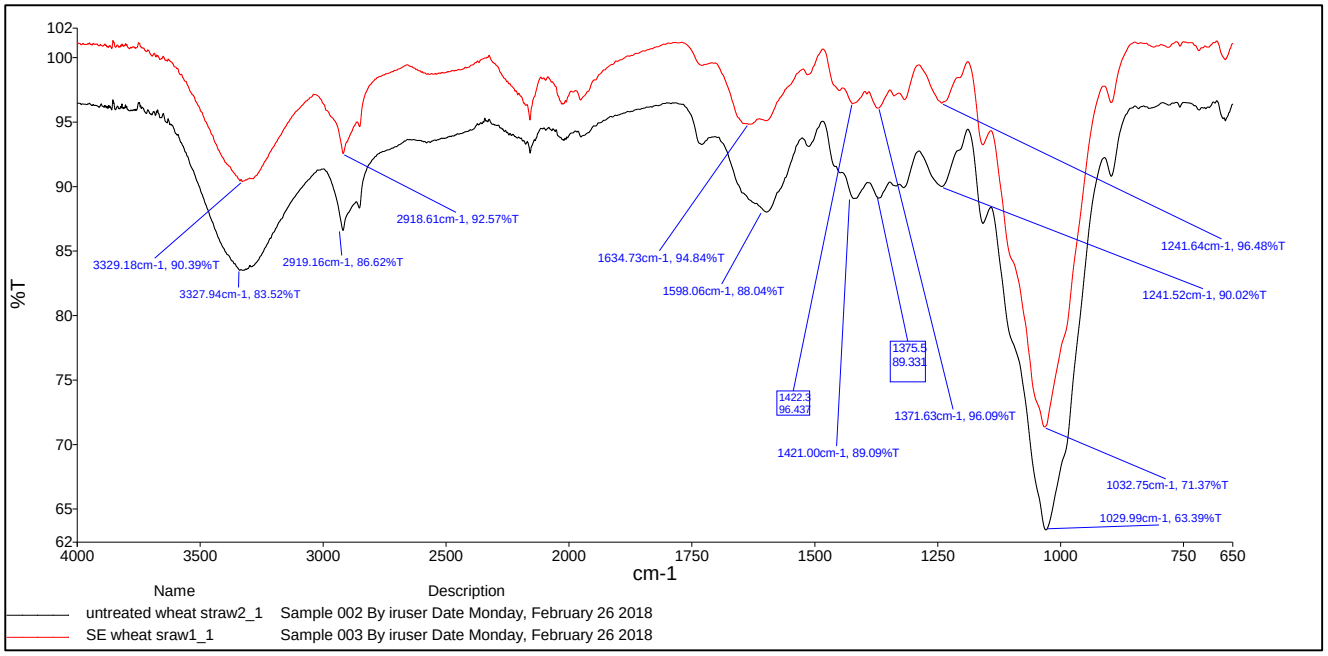


Figure 3-2: The FTIR spectra for the untreated (WS) and the steam-exploded straw (SE) which used as a feedstock for the CSTR experiment

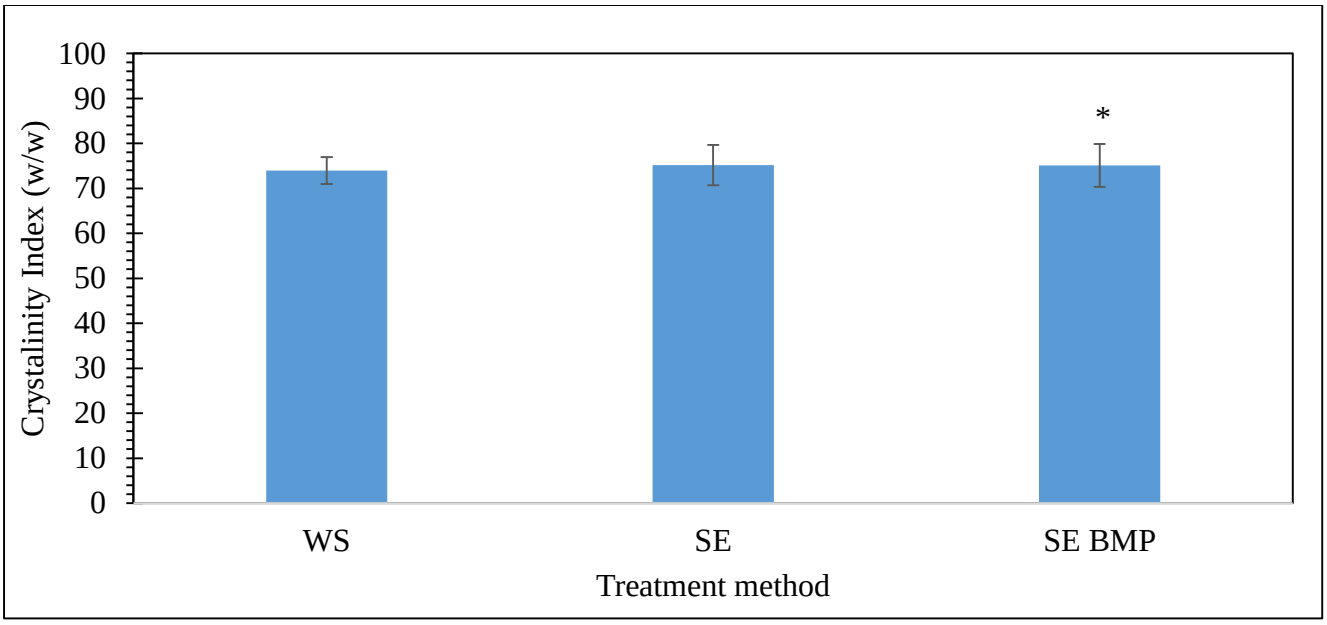


Figure 3-3: Crystallinity index within the untreated WS used as a feedstock in the bioreactors and steam exploded at a temperature of 160 °C and pressure of 6.4 bar straw samples * The SE BMP represents the average results from all the 13 different steam-exploded

3.5.4 BMP experiment comparing the biogas yields from different steam explosion pretreatments

The cumulative methane production was calculated for the different steam-exploded and untreated WS samples after 30 days of digestion, and results are presented in Fig. 3-4. As a general remark, the untreated WS samples produced lower amounts of gas compared to the steam-exploded straw samples but the increase that the pretreatment offered was significant only for some of the examined pretreatment trials. The methane production from the untreated straw samples (WS_1, WS_2 and WS_3) ranged between 237 and 281 mL CH_4/g VS, whereas for SE methane production ranged from 215 to 338 ml CH_4/g VS. The remarkable differences in the sugars concentration can explain to an extent the alternations on the biomethane production from the different untreated samples. In total, seven out of thirteen pretreatments seemed to improve biomethane production compared to WS_1, which was also the starting material used to produce all steam-exploded samples. Against WS_2, only four pretreatments enhanced gas yields, while the statistical analyses also show that none of the pretreatments offered statistically increased yields when compared to the yields from WS_3. On the contrary, the use of SE_{18} (P=5.8 bar and T=163°C) provided significantly lower amounts of gas ($p<0.05$) compared to both WS_2 and WS_3. Concerning the biomethane production among SE feedstock, SE_{04} (SF 2.9), SE_{06} (SF 3.05) and SE_{17} (SF 2.76) offered significantly higher methane yields compared to the yields offered by the rest of the pretreated samples ($p<0.05$). It seems that a lower severity factor in SE treatment (2.76 for SE_{17}) was more beneficial towards the increase in the biomethane yields compared to performance after SE treatment with higher intensity (2.91 in SE_{04} and 3.05 in SE_{06}). However, further decreased in the S.F. in the case of SE_{18} caused a significant reduction in the methane yields achieving levels lower than two of the untreated samples (WS_2

and WS_3) (Fig.3-4). More work needs to be conducted for identifying the optimal steam explosion pretreatment conditions towards the increase of WS's biomethanation.

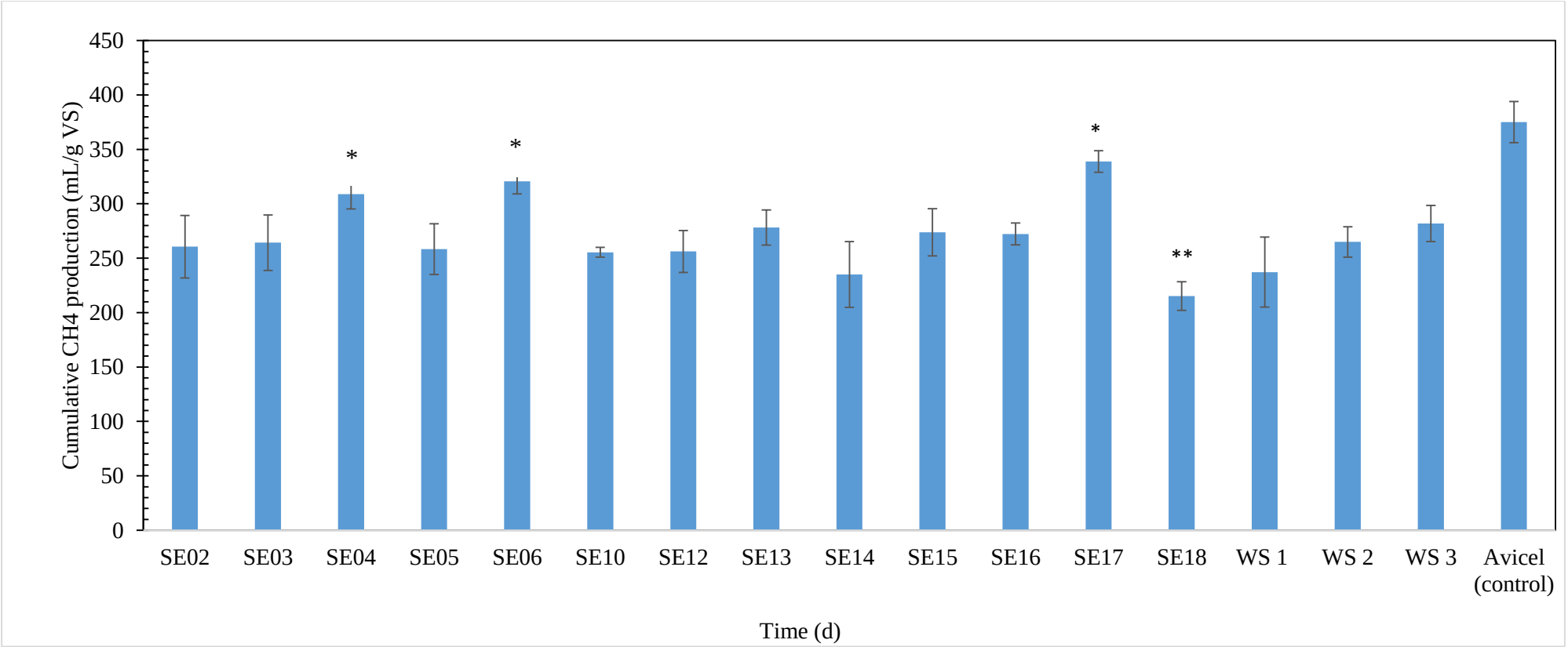


Figure 3-4: Cumulative CH_4 yields for steam-exploded and untreated WS samples, after a digestion period of 30 days. The one asterisk symbol (*) indicates the significant difference in the methane production compared to WS_1, WS_2 and WS_3. The two asterisks (**) indicate a significant lower methane production from the WS_2 and WS_3

Measurements of the VS content of the influent and the effluent of the BMP experiment were taken to test whether SE affected the density inside the AD reactor. A statistically significant decrease in the VS content was found for all the examined steam explosion pretreatment conditions as presented in Fig. 3-5. This is a promising outcome as the increased density in AD reactors digesting WS is a common problem for CSTR systems. However, none of the treatments seemed superior to the rest regarding the reduction in the solids content of the reactor. Unlike the cases of the steam-exploded feedstock, the VS content in the vials digesting untreated WS was not significantly reduced in any of the examined samples (Figure 3-5). Probably, a slightly longer digestion period would be required in order for the microorganisms of the system to be able to digest the lignocellulose to a higher extent and so offer higher gas yields when WS is digested. These results can prove that the hydrolytic activity of the microorganisms of AD was enhanced when a steam explosion pretreatment stage was previously applied.

An important indication of efficient digestion for all feedstock tested in this BMP trial is below the detection of the HPLC limit accumulation of VFAs at the end of the experiment. Furthermore, the pH was measured within the range 7,1-7,8 for all BMP trials during the whole experimental period while these values lying within the optimal range for AD systems as described by Angelidaki and Sanders (2004). Data related to the VFAs accumulation and the pH measurement on the BMP trials are not presented.

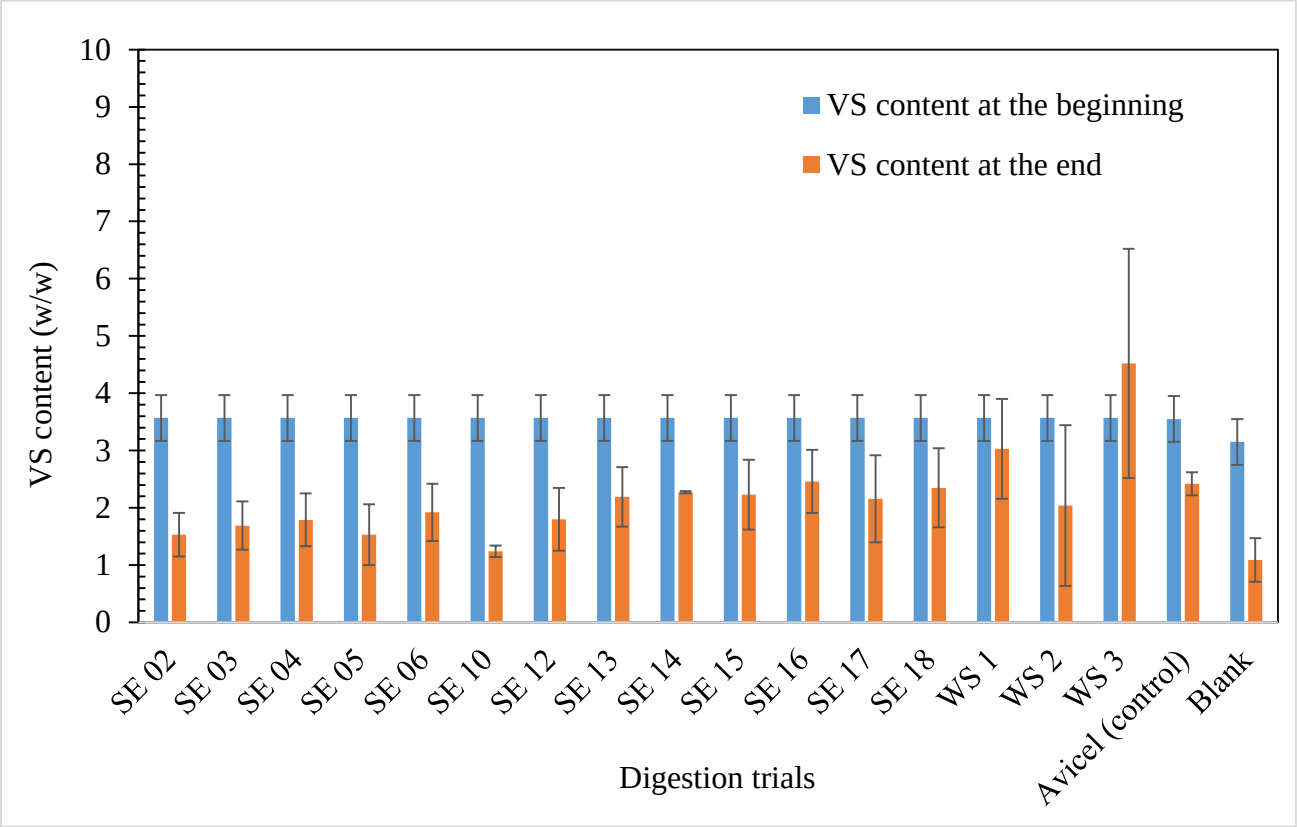


Figure 3-5: The % volatile solids (VS) content measured on the different BMP trials at the beginning and after the end BMP experiment. The VS content at the beginning of the experiment is calculated as the sum of the VS content from the inoculum and each feedstock

3.5.5 Comparison of continuous digestion of WS and SE

An additional experiment was carried out, aiming to evaluate the effect of steam explosion pretreatment of WS on the continuous AD process. As such, a pair of CSTR bioreactors operated with untreated straw (WS) while another pair operated with SE pretreated at a temperature of 160 °C and pressure of 6.4 bars (SF 2.9). The average daily methane production from each one of the two bioreactors pairs, along with the reported deviations, are presented in Fig. 3-6. The system operated for a total period of 209 days with relatively stable methane production, stable pH and low accumulation of VFAS, with however two short break periods (days 55-61 and 73-79) when engineering

problems were limiting its efficiency. Those issues were related to unexpected general power shutdowns, which also created problems with the heating and mixing of the system, as well as issues related to the blockages of the tubings. The experimental period can be divided into two sub-periods based on the OLR that was used on each occasion. Sub-period A (days 0-107) represents the starting OLR of 2 g VS/L day^{-1} while subsequently, for sub-period B (days 108-209) the OLR was increased to 5 g VS/L day^{-1}. The OLR increased gradually over three days in order to avoid providing the system with further stress of very rapid production of AD intermediate by-products (e.g. VFAs) which might have caused a significant decrease in the pH (Rajput and Sheikh, 2019) and eventually a total inhibition to the system. However, the increase in the ORL and the stress that this created on the bioreactor can explain a temporary drop in the methane production for both feedstocks on day number 109, immediately after the increase in OLR. In general, the system seemed to be robust and demonstrated a fast adaptation to alterations related either to differences in the temperature, unexpected power shutdowns or other technical limitations. An average increase of 20% (v/v) on the biomethane yields was reported for the reactors digesting SE compared to the reactors digesting untreated WS. Regarding the effect of the increased OLR on the efficiency of the system, after a steady-state operation was reached, no significant changes were reported on the methane yields between the two periods for both WS and SE. For the whole experimental period, the WS offered daily on an average level of 215 ml/g VS biomethane while the SE produced almost 20% more (270 ml/gVS). The increase in the produced gas yields due to the pretreatment, for both examined OLR, was within the limit reported in the BMP experiment of the present study but also is equivalent to the increase reported in previous studies examining different hydrothermal pretreatments on WS (Bauer et al., 2009; L.C. Ferreira et al., 2014). The

system demonstrated a relatively fast adaptation to the increased OLR so a further increase can also be considered as future work. Pohl et al., (2013) reported that for an OLR higher than 8 gVS/L day^{-1} the AD of WS has to be conducted in a two digestion system due to limitations related to the fast production of VFAs. However, Pohl and his team worked under thermophilic conditions where the production of VFAs is faster compared to the mesophilic systems. As a consequence, an increase of the OLR to levels higher than the above threshold might be possible in order to increase the biomethane production from WS.

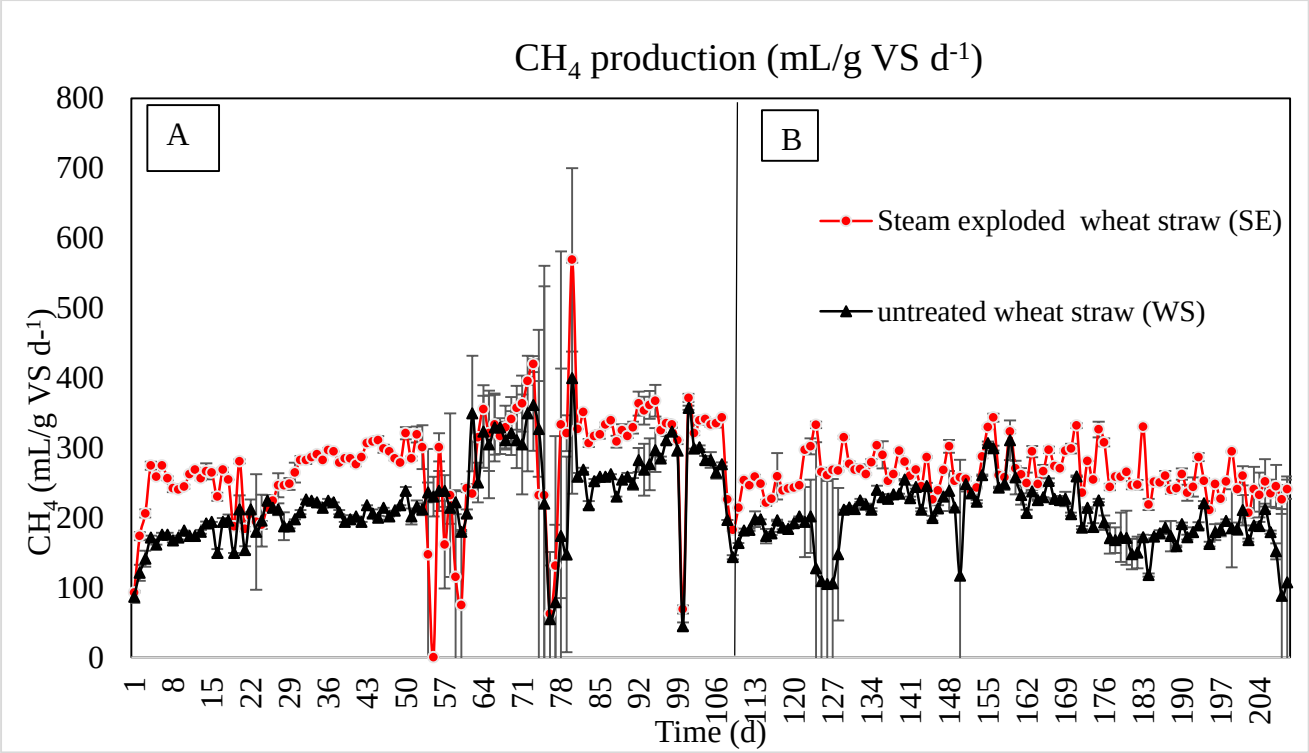


Figure 3-6: Average daily biomethane production from untreated and steam-exploded wheat straw

In addition to the relatively stable methane production, the accumulation of the VFAs in all four bioreactors was continuously close or below the HPLC detection limits (0.05mg/l) with only a few occasions in which VFA's exceed the concentration of 1 g/L (Fig. 3-7) while acetic acid (AA) was found in the highest concentrations compared

to the rest of the VFAS. The reported values can be considered within the limits for AD running on lignocellulosic feedstock. It was already stated in the past that similar digestion systems found with concentrations even at a level of 10g/L while they were able to recover and operate again under steady-state (Rouches et al., 2019). Concerning the pH of each bioreactor, a decreasing trend was observed without this significantly affecting the biomethane production. Similar to the BMP results where the pH never found below 7,2, the pH in all four bioreactors (Figure 3-8) never exceeded the acceptable values for AD (Angelidaki and Sanders, 2004). Finally, the total solids content from the bioreactors effluents followed an increasing trend, with average values from 8 g TS/kg to higher than 12 g TS/kg (figure 3-9). This accumulation of the feedstock inside the digesters can potentially create problems with the mixing system. A potential solution for this problem would be either the reduction of the daily feedstock or the addition of extra amounts of water in the digester.

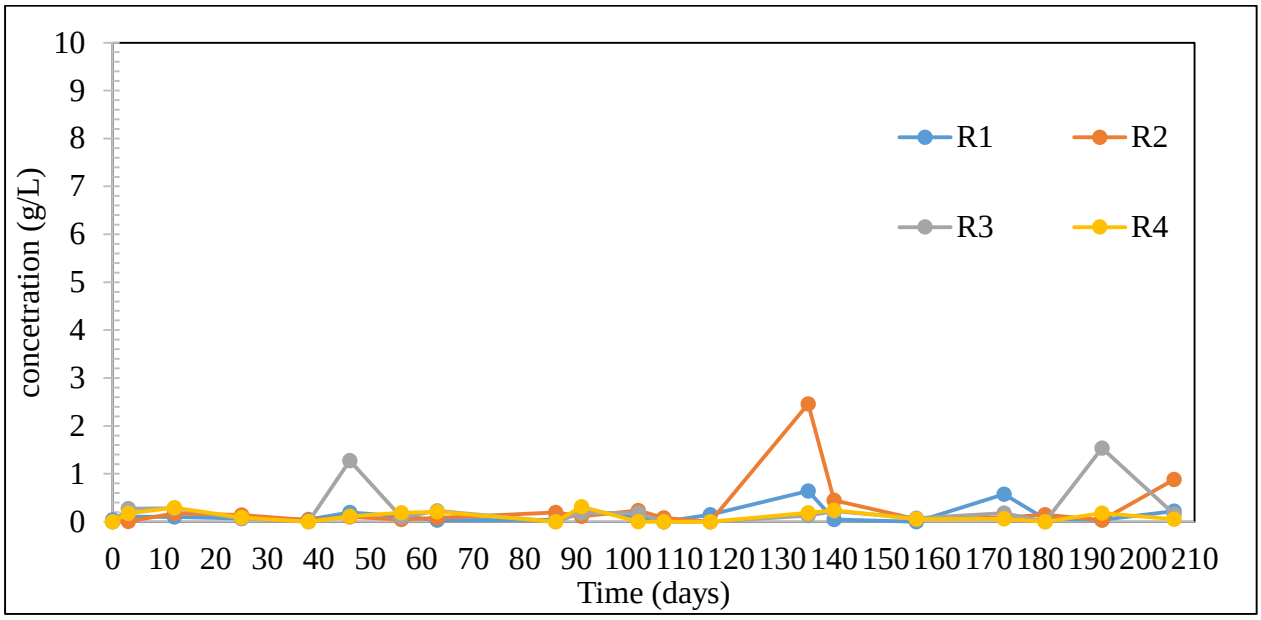


Figure 3-7: Total VFAs profile for the entire experimental period for digesters R_1 and R_2 digesting SE and R_3 and R_4 digesting WS (control)

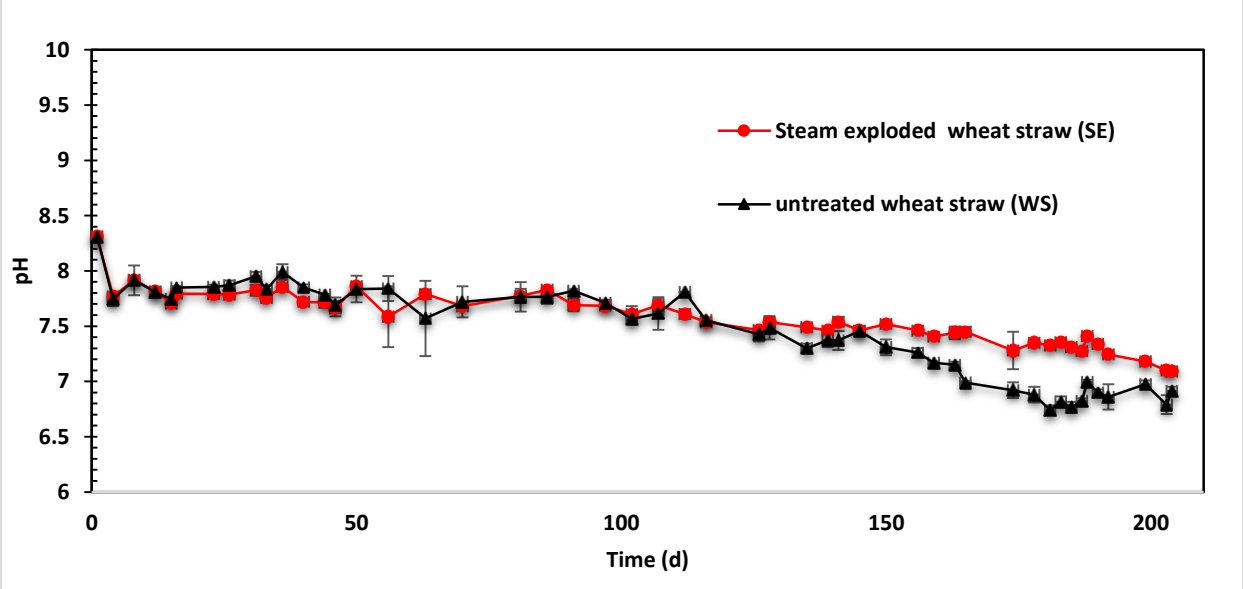


Figure 3-8: Average pH values for each bioreactor's pair digesting steam-exploded (R_1 and R_2) and untreated (R_3 and R_4) wheat straw

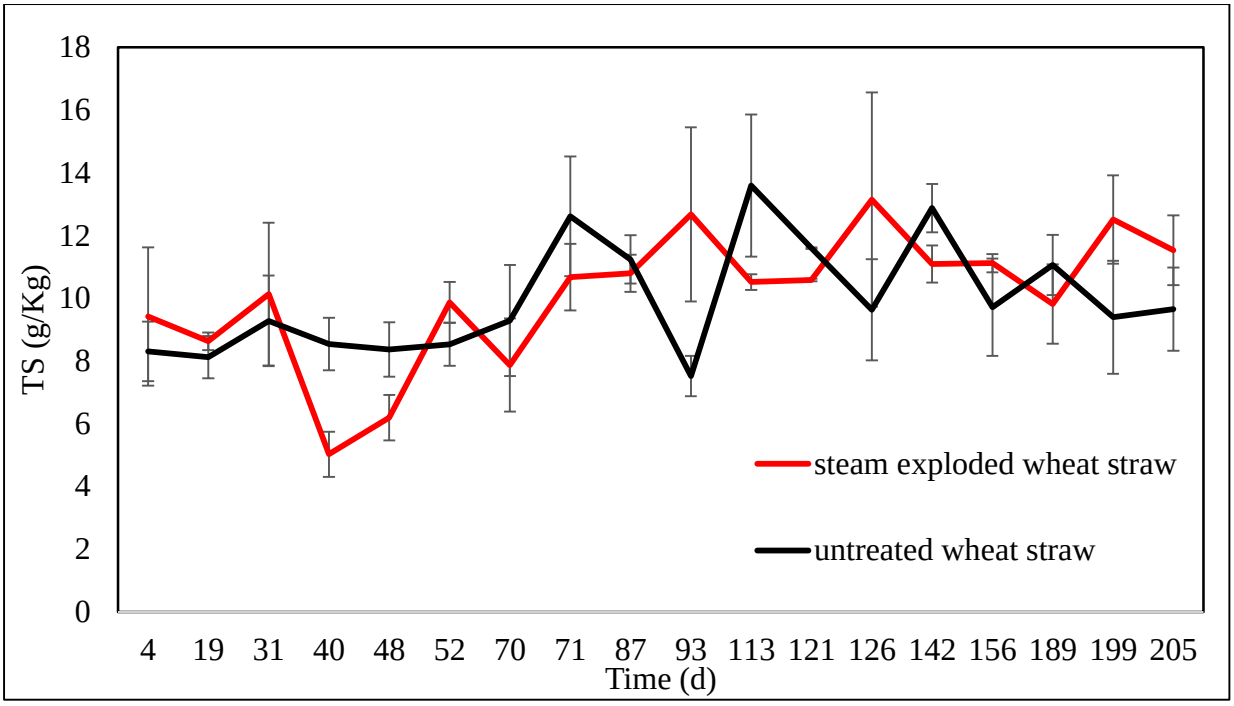


Figure 3-9: The average total solids (TS) content on the effluent of the bioreactors fed with untreated (WS) and the steam-exploded straw (SE)

3.5.6 Microbiological analysis

The diversity of the populations of microorganisms inside the four bioreactors during the CSTR experimental period is presented as a Shannon diversity index (H) value in Fig. 3-10. This index can be used as a method to categorise different microbial communities, including an AD system, based on the richness of their biodiversity (Veech, 2017). In the present study, the two different systems (one digesting WS and the other one digesting SE) seemed to have similar microbial richness for the whole experimental period, indicating an insignificant effect of the steam explosion pretreatment on the microbial diversity of AD. An exception to this was the point in AD fermentation that the OLR was increased (day 108), after which, the H values were decreased for the two reactors digesting SE (R_1 and R_2) but remained relatively stable for R_3 and R_4 where WS was fed. This might be an indication that the AD system fed with SE is more sensitive to changes in the reactor's operation compared to the system

fed with WS. It is possible that the increased presence of VFAs in the reactors digesting SE compared to the ones digesting WS is the reason for these results. However, the system proved to be robust while the richness of the species in R_1 and R_2 was quickly restored to values similar to the ones recorded before the increase in the OLR. At the same time, for the cases of R_3 and R_4, the H value also seemed to slightly decrease after the increase in the OLR, in a delayed fashion compared to reactors R_1 and R_2. A recently published work from Braz et al. (2019) who worked on sewage sludge examined the effect of a sudden increase of the OLR on the microbial populations of AD systems. Braz et al., (2019) reported that more work will be required in order to understand the several mutualistic, as well as competitive interaction, occurred between the different species of the system. In the same study, it was also found a temporal succession of some minor importance for the system microorganisms after the increase in the OLR.

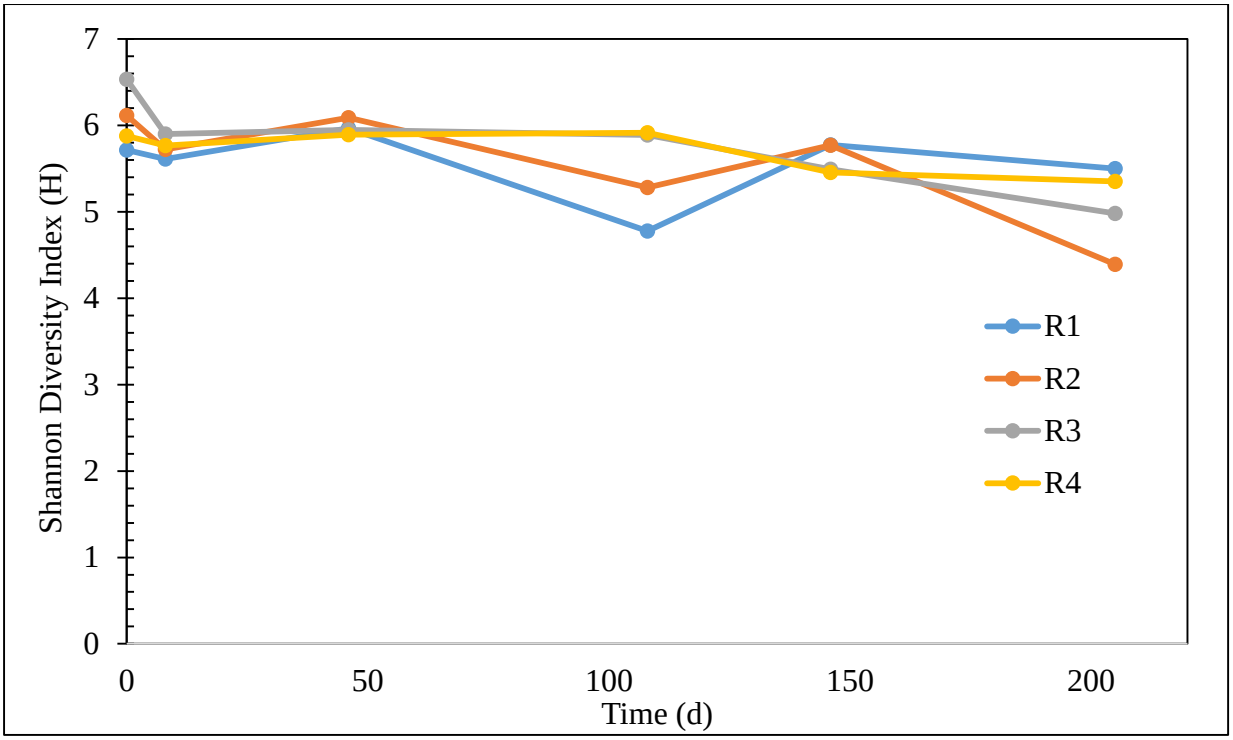


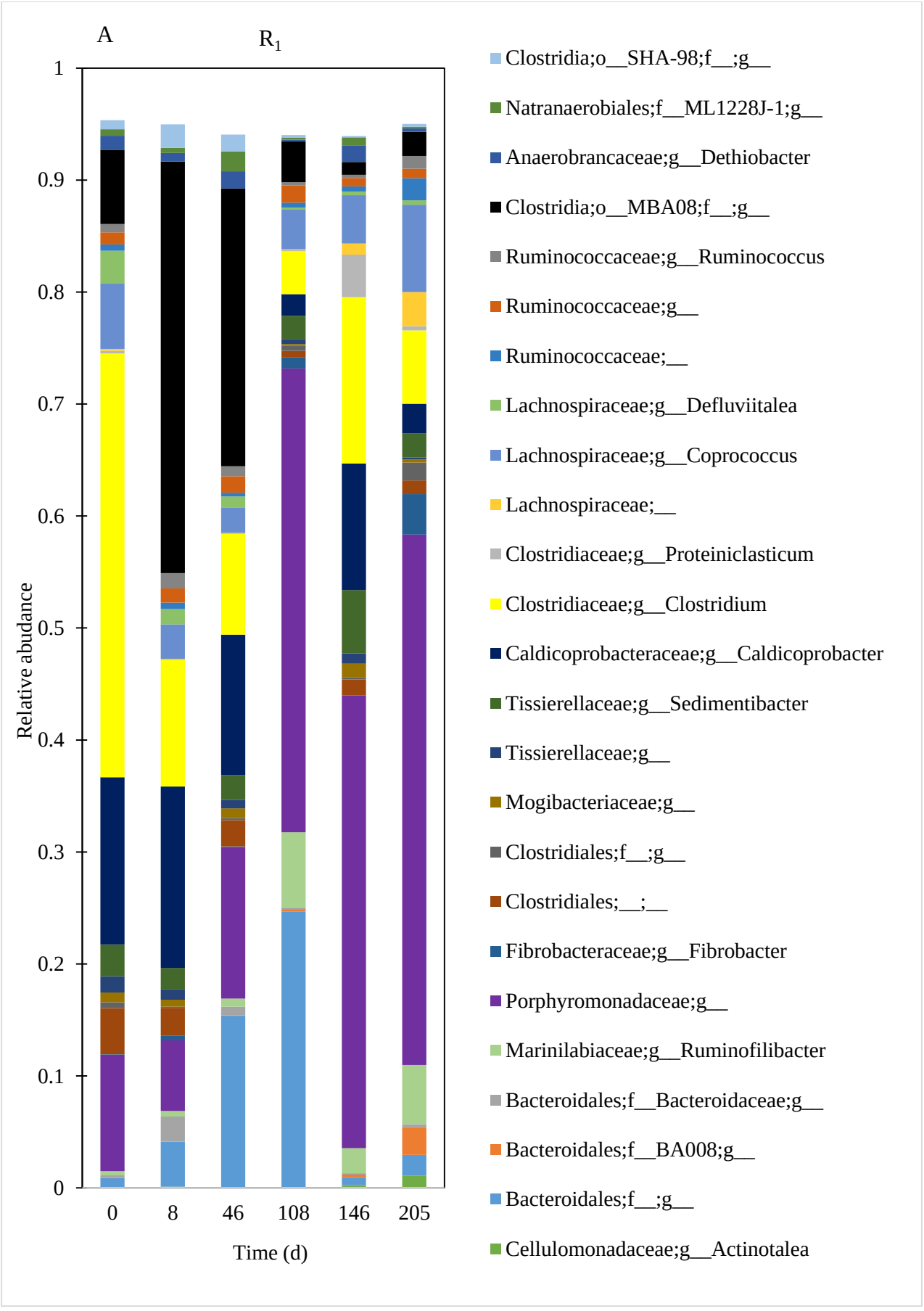
Figure 3-10: Shannon diversity index (H) for bioreactors digesting steam-exploded straw (SE) (R_1 and R_2) or untreated wheat straw (WS) (R_3 and R_4)

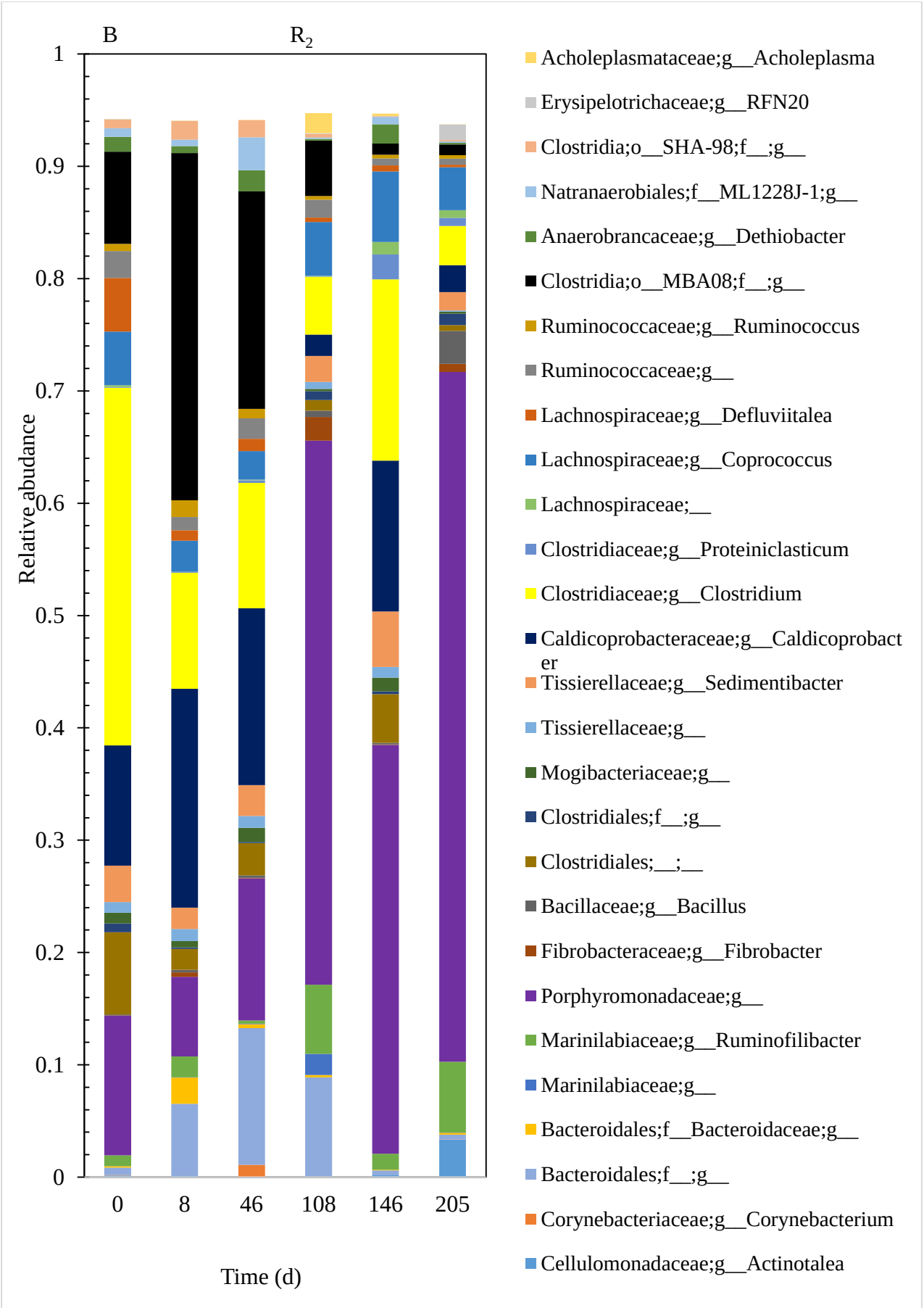
3.5.6.1 Bacterial communities

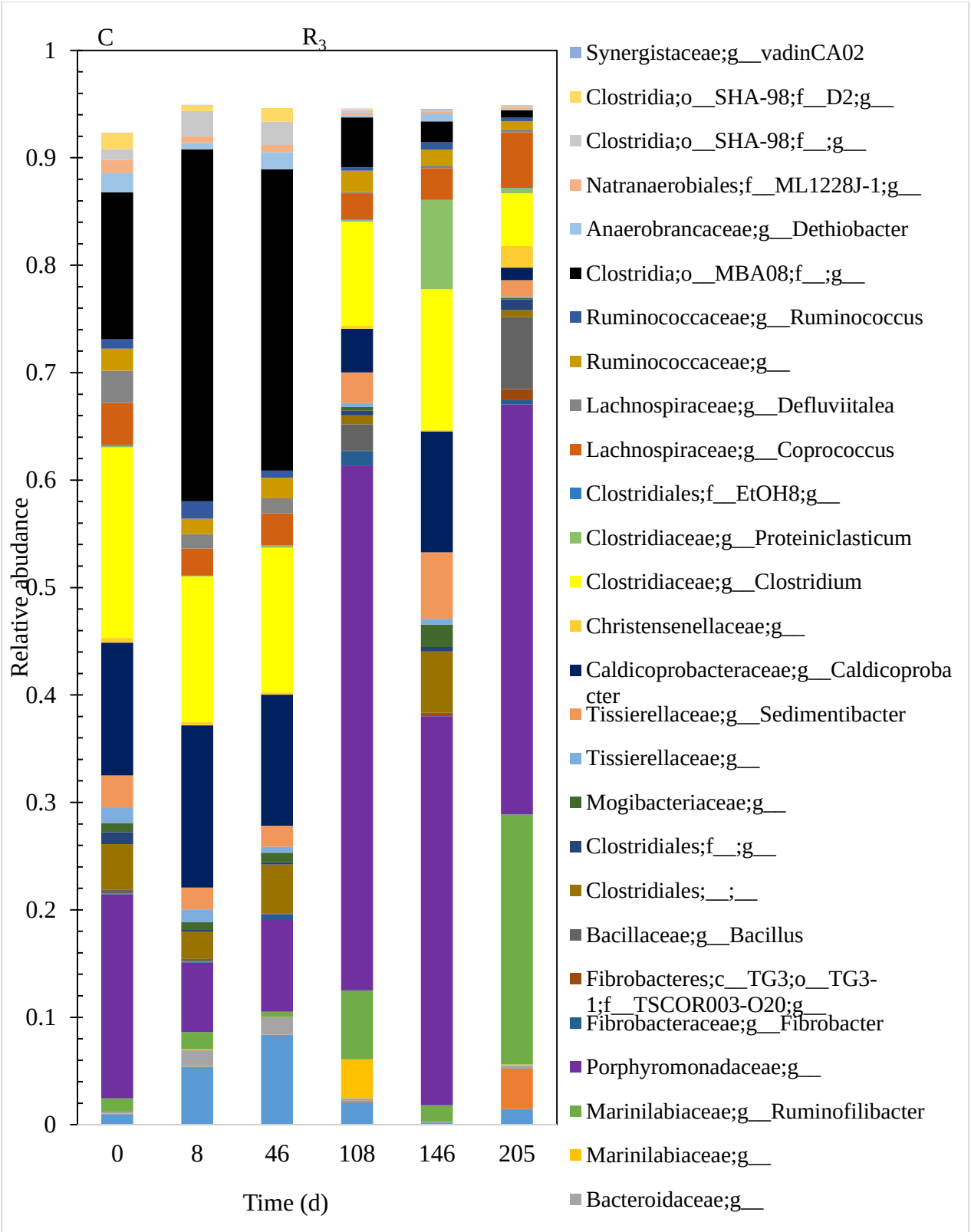
Apart from the richness of the microbial biodiversity, the relative abundances for the species found inside the four reactors also seemed to be more affected by the OLR increase rather than the difference of the feedstock (Figure 3-11 A-D). A shift, regarding the different microbial abundances, was observed for all four reactors after the increase of the OLR. More specifically, the genus *Clostridium* sp. was the most abundant in all reactors at the beginning of the digestion period (day 0). In previous work, *Clostridium* sp. was found to conduct the breakdown of cellulose and hemicellulose through the production of cellulosome which is a multi-enzyme extracellular complex (Zhang et al., 2015). The reported differences in the microbial populations in the inoculum of the four systems (day 0) can be explained since all bioreactors were operating for a while (pre-digestion period) under the same feedstocks

before the beginning of the experiment. The operational conditions for the bioreactors during this pre-experimental period were also the same as the ones used during the beginning of the experiment. Before the starting of the CSTR experiment, all reactors were left to starve for 10 days and this was considered the inoculum for the experiment. Furthermore, for all bioreactors, *Clostridium* sp. seemed to decrease after day 8. Next, during days 8 and 46 another clostridia species with an unidentified family (*Clostridia__MBA08*) was found in the highest concentration, ranging between 25-40% (w/w) in all bioreactors. In previous studies where the AD of straw was evaluated this microbial species (*Clostridia__MBA08*) was also found in high concentrations (Sun et al., 2015). The next sampling day was after the increase in the OLR of the system (Day 108) while the concentration of both clostridia families seemed to be reduced after this change. A similar reaction to the increase of the OLR was reported also for the microorganisms that belong to genus *Caldicoprobacter* sp. while its presence reached levels up to 20% (w/w) before the increase in the feeding rate and almost disappeared from the reactors after day 108. *Caldicoprobacter* sp, which is also a member of the family of *Clostridiales*, has previously been found to degrade sugars (Amel et al., 2016; Bouacem et al., 2015) and proteins (Bouacem et al., 2015). This is strange considering the fact that after the increase in the OLR higher amounts of sugars were expected inside the bioreactors. On the other hand, after the increase of the OLR (days 146 and 205), a rapid increase in the population of *Porphyromonadaceae* was observed, accounting for 35-60% (w/w) of the total bacterial population. According to Martinez-Burgos et al., (2020), different genera that belong to this family are *Porphyromonas, Odoribacter, Butyricimonas, Parabacteroides, Paludibacter, Tannerella Petrimonas, Proteiniphilum* and *Macellibacteroides*. The metabolism of this type of microorganisms that belong to the order of *Bacteroidales* is also linked with the

degradation of carbohydrates (Dong et al., 2019) and the VFA's utilization (Poszytek et al., 2017). It is possible that more rapid production of AD's intermediate products due to the increased OLR caused this shift in the microbiology of the system and the low accumulation of VFAs can be attributed to the increased presence of the family of *Porphyromonadaceae*. However, the stable pH of the system in accordance with the VFA's profile for the whole experimental period indicates that the system was able to biologically tolerate the increased OLR. Finally, bacteria that belong to the genus of *Ruminofilibacter* sp. and are associated with the degradation of cellulose (Dong et al., 2019), were initially present in the reactors in very small quantities at the beginning of the experiment, and reached concentrations of 23% and 40% (w/w) for R_3 and R_4 at day 205.


A
R1
Relative abudance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
8
46
108
146
205
Time (d)
Clostridia;o__SHA-98;f__;g__
Natranaerobiales;f__ML1228J-1;g__
Anaerobrancaceae;g__Dethiobacter
Clostridia;o__MBA08;f__;g__
Ruminococcaceae;g__Ruminococcus
Ruminococcaceae;g__
Ruminococcaceae;__
Lachnospiraceae;g__Defluviitalea
Lachnospiraceae;g__Coprococcus
Lachnospiraceae;__
Clostridiaceae;g__Proteiniclasticum
Clostridiaceae;g__Clostridium
Caldicoprobacteraceae;g__Caldicoprobacter
Tissierellaceae;g__Sedimentibacter
Tissierellaceae;g__
Mogibacteriaceae;g__
Clostridiales;f__;g__
Clostridiales;__;__
Fibrobacteraceae;g__Fibrobacter
Porphyromonadaceae;g__
Marinilabiaceae;g__Ruminofilibacter
Bacteroidales;f__Bacteroidaceae;g__
Bacteroidales;f__BA008;g__
Bacteroidales;f__;g__
Cellulomonadaceae;g__Actinotalea


B
R2
Relative abudance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
8
46
108
146
205
Time (d)
Acholeplasmataceae;g__Acholeplasma
Erysipelotrichaceae;g__RFN20
Clostridia;o__SHA-98;f__;g__
Natranaerobiales;f__ML1228J-1;g__
Anaerobrancaceae;g__Dethiobacter
Clostridia;o__MBA08;f__;g__
Ruminococcaceae;g__Ruminococcus
Ruminococcaceae;g__
Lachnospiraceae;g__Defluviitalea
Lachnospiraceae;g__Coprococcus
Lachnospiraceae;__
Clostridiaceae;g__Proteiniclasticum
Clostridiaceae;g__Clostridium
Caldicoprobacteraceae;g__Caldicoprobacter
Tissierellaceae;g__Sedimentibacter
Tissierellaceae;g__
Mogibacteriaceae;g__
Clostridiales;f__;g__
Clostridiales;__;__
Bacillaceae;g__Bacillus
Fibrobacteraceae;g__Fibrobacter
Porphyromonadaceae;g__
Marinilabiaceae;g__Ruminofilibacter
Marinilabiaceae;g__
Bacteroidales;f__Bacteroidaceae;g__
Bacteroidales;f__;g__
Corynebacteriaceae;g__Corynebacterium
Cellulomonadaceae;g__Actinotalea


C
R3
Relative abudance
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
8
46
108
146
205
Time (d)
Synergistaceae;g__vadinCA02
Clostridia;o__SHA-98;f__D2;g__
Clostridia;o__SHA-98;f__;g__
Natranaerobiales;f__ML1228J-1;g__
Anaerobrancaceae;g__Dethiobacter
Clostridia;o__MBA08;f__;g__
Ruminococcaceae;g__Ruminococcus
Ruminococcaceae;g__
Lachnospiraceae;g__Defluviitalea
Lachnospiraceae;g__Coprococcus
Clostridiales;f__EtOH8;g__
Clostridiaceae;g__Proteiniclasticum
Clostridiaceae;g__Clostridium
Christensenellaceae;g__
Caldicoprobacteraceae;g__Caldicoproba cter
Tissierellaceae;g__Sedimentibacter
Tissierellaceae;g__
Mogibacteriaceae;g__
Clostridiales;f__;g__
Clostridiales;__;__
Bacillaceae;g__Bacillus
Fibrobacteres;c__TG3;o__TG3-1;f__TSCOR003-O20;g__
Fibrobacteraceae;g__Fibrobacter
Porphyromonadaceae;g__
Marinilabiaceae;g__Ruminofilibacter
Marinilabiaceae;g__
Bacteroidaceae;g__

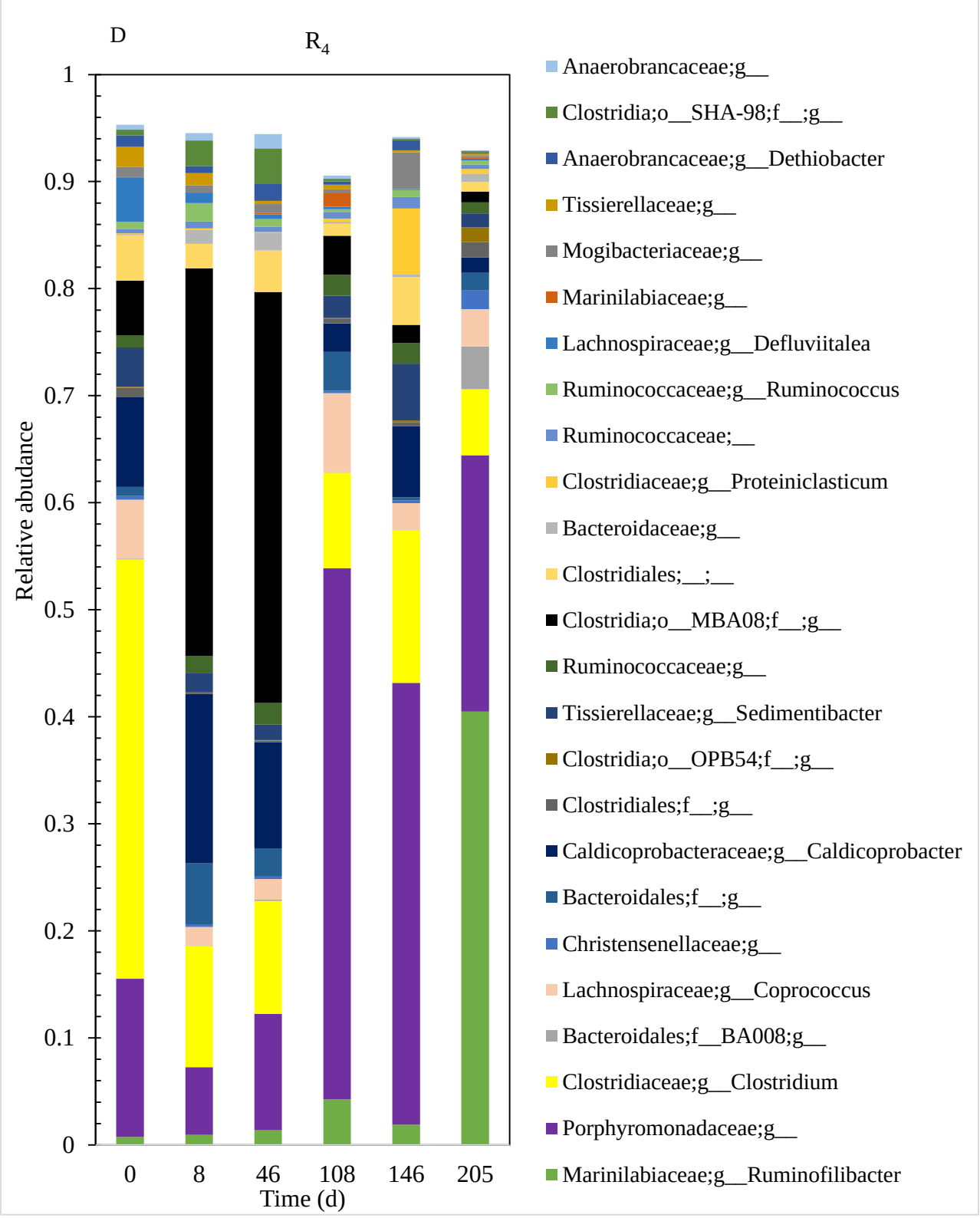


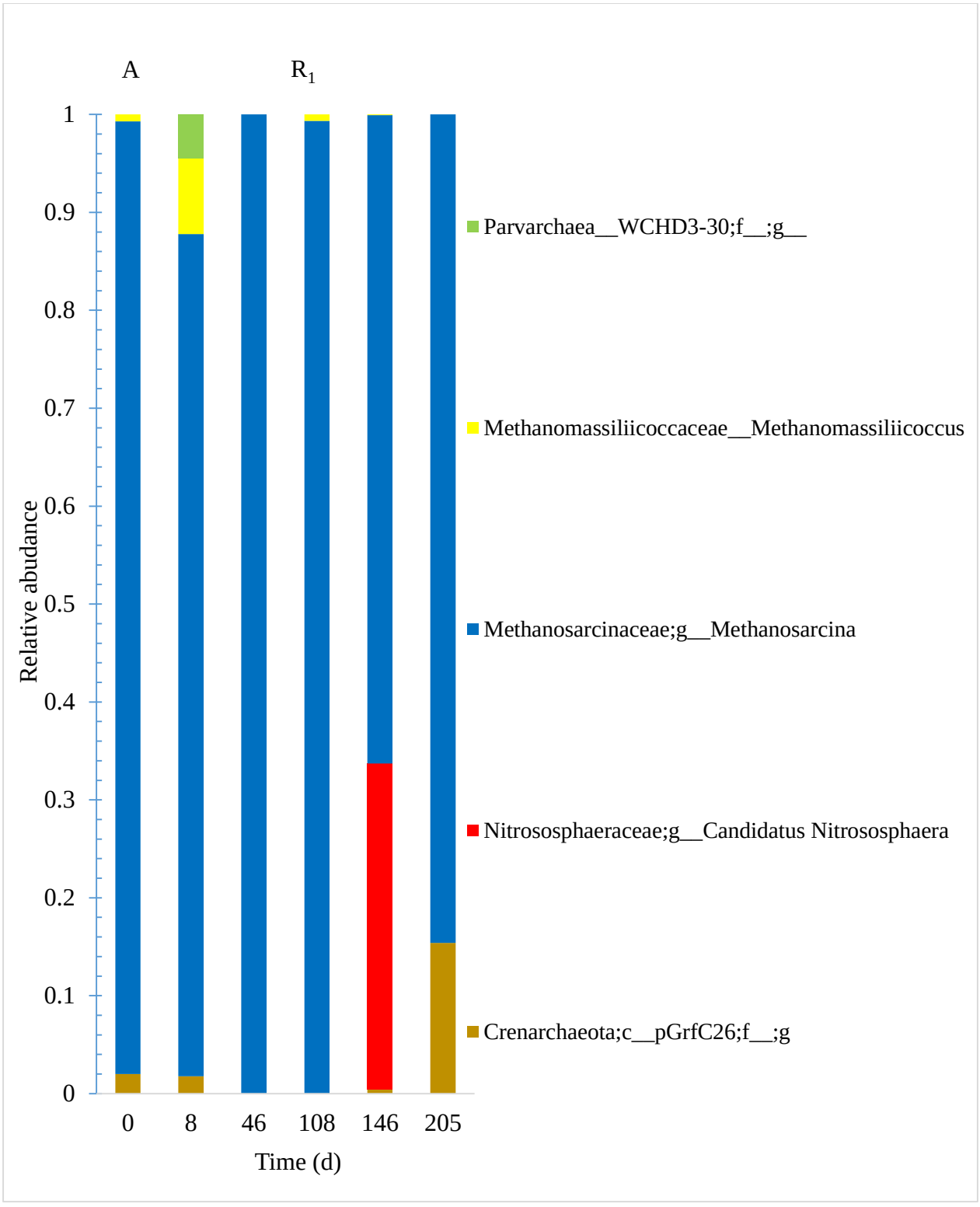
Figure 3-11: Composition of bacteria at the genus level in the anaerobic digestion system at bioreactors. A and B: $R_1$and R_2 respectively digesting steam-exploded wheat straw (SE). C and D: R_3 and R_4 digesting untreated wheat straw (WS)

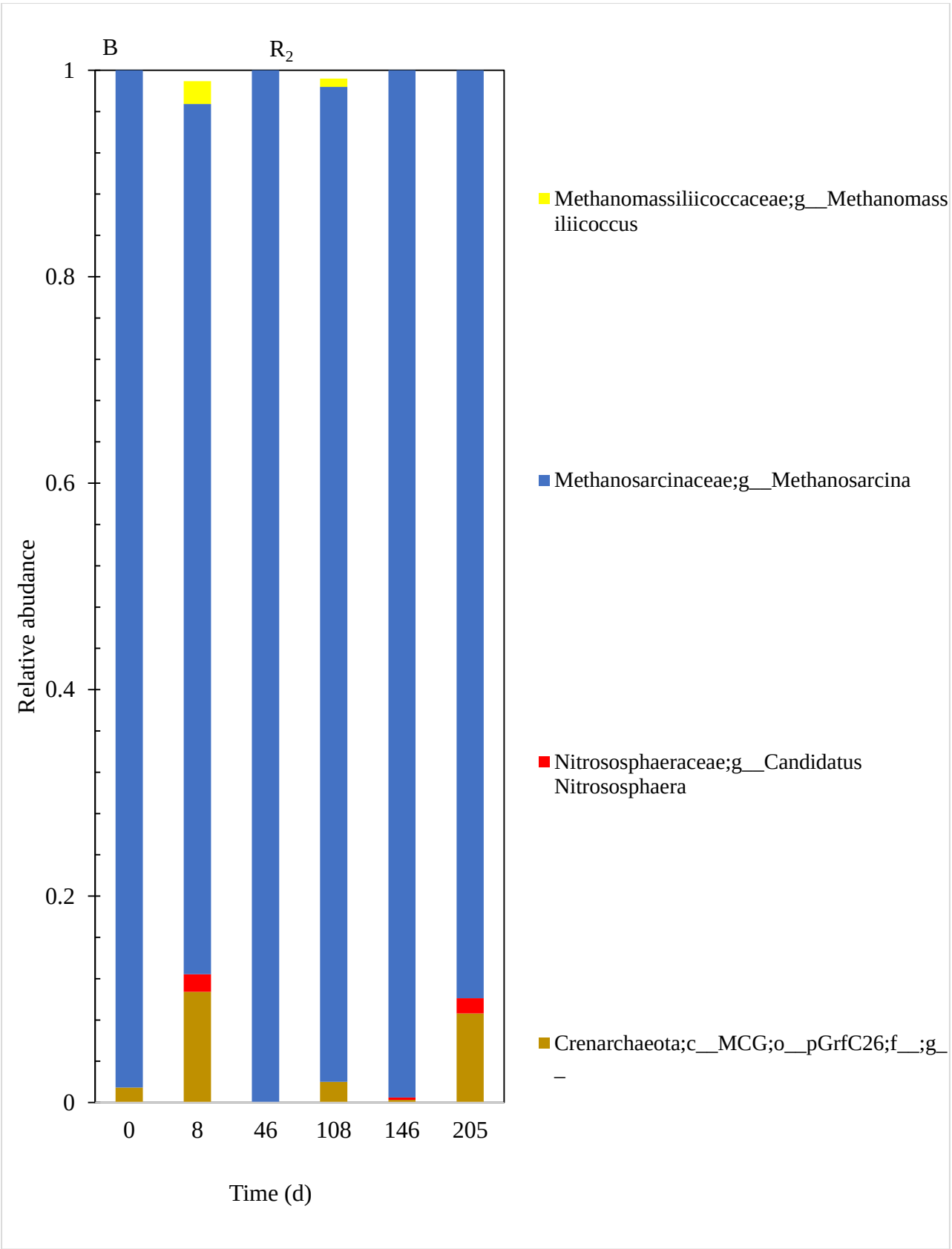
3.5.6.2 Archaea composition

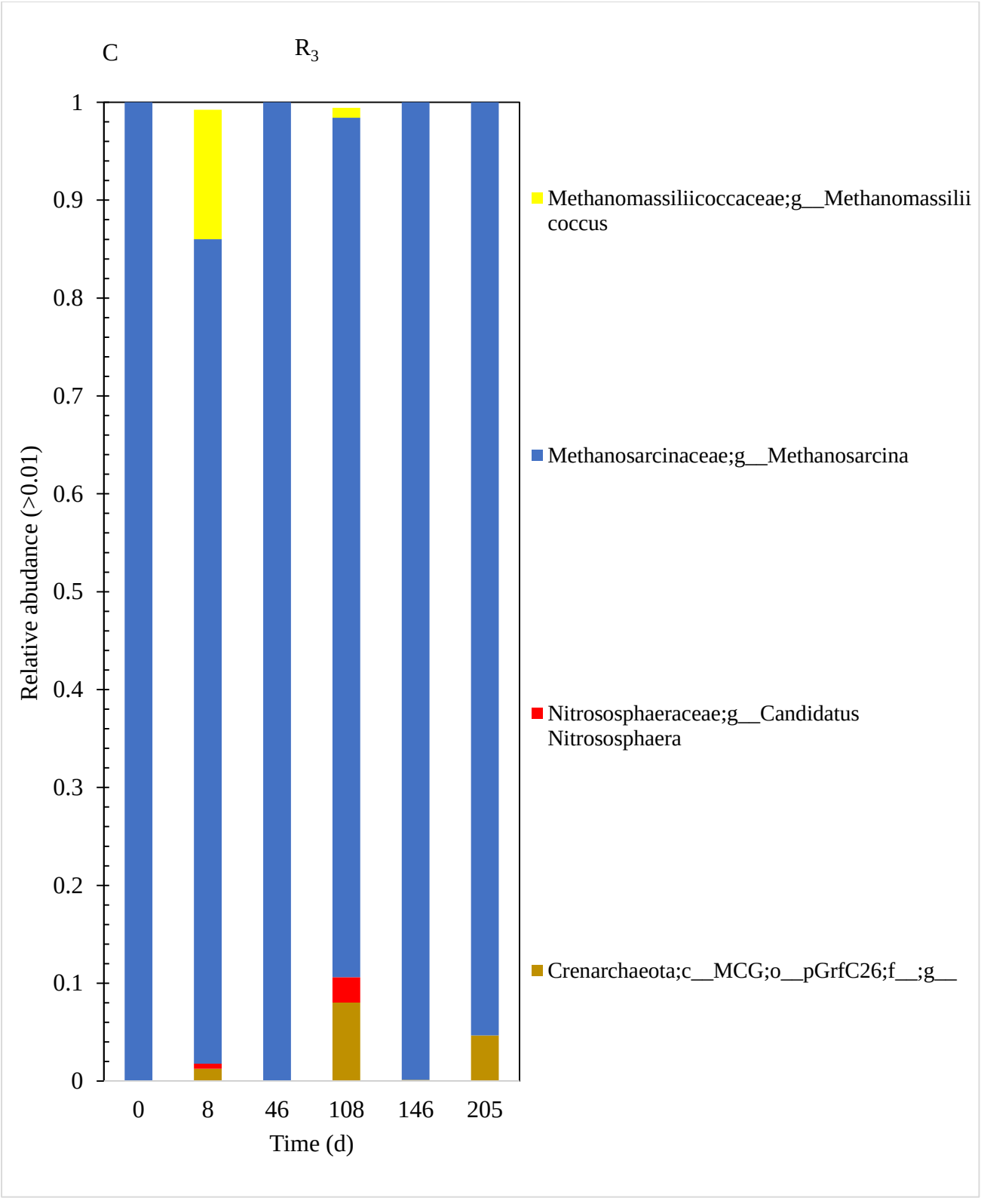
Apart from the bacterial populations, the present study also examined the effect of steam explosion pretreatment of WS on the methanogenic populations of AD (figure 3-12 A-D). The biodiversity of archaebacteria was limited, whereas a maximum of five species was found, in concentrations higher than 1% (w/w) of the total archaea population, per reactor. In all four reactors, the most dominant species found was *Methanosarcina spp* which in some cases was found to hold a percentage up to 98% (w/w) of the total archaea population. *Methanosarcina spp* is already known for its ability to convert acetate to methane (Lins et al., 2014), through acetotrophic methanogenesis. As a consequence, it is believed that high concentrations of acetic acid where produced during the experimental period but they were subsequently consumed by this type of microorganism. Apart from *Methanosarcina spp,* an unspecified Crenarchaeota species and the methanogen *Methanomassiliicoccus* sp. were counted at levels lower than 10% (w/w) of the total population. Finally, members of the family *Nitrososphaeraceae,* which are known for their ability to oxidize ammonia (Pelissari et al., 2017), were found in different concentrations inside all bioreactors. Interestingly, the highest concentration for this species (33% (w/w)) was found at day 146 and reactor R_1 while in R_2, the population of this microorganism never exceeded the threshold of 2% (w/w). Studies have reported that the ammonia utilising *Nitrososphaeraceae* clusters are alkaliphilic microorganisms and they grow better under increased availability of ammonia and high pH levels. However, in the present study, no significant increase in the pH levels or other sign of inhibitions was reported for R_1 at day number 146.

In general, the microbiological analyses from the present experiment revealed a potential correlation between the OLR in an AD system and the microbial populations

that colonise it. However, the limited knowledge regarding the synergistic and/or competitive interaction between the microorganisms of the system along with a general lack of information regarding several metabolic capabilities of these microorganisms are limiting the possibilities for conclusive outcomes. In that direction, in the future, further investigation on the role of individual microorganisms on AD will be required in order to understand in depth the behaviour of the system to several alternations of its operational parameters.


A
R1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative abudance
0
8
46
108
146
205
Time (d)
Parvarchaea__WCHD3-30;f__;g__
Methanomassiliicoccaceae__Methanomassiliicoccus
Methanosarcinaceae;g__Methanosarcina
Nitrososphaeraceae;g__Candidatus Nitrososphaera
Crenarchaeota;c__pGrfC26;f__;g


B
R2
1
0.8
0.6
0.4
0.2
0
Relative abudance
0
8
46
108
146
205
Time (d)
Methanomassiliicoccaceae;g__Methanomassiliicoccus
Methanosarcinaceae;g__Methanosarcina
Nitrososphaeraceae;g__Candidatus Nitrososphaera
Crenarchaeota;c__MCG;o__pGrfC26;f__;g__


C
R3
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Relative abudance (>0.01)
0
8
46
108
146
205
Time (d)
Methanomassiliicoccaceae;g__Methanomassiliicoccus
Methanosarcinaceae;g__Methanosarcina
Nitrososphaeraceae;g__Candidatus Nitrososphaera
Crenarchaeota;c__MCG;o__pGrfC26;f__;g__

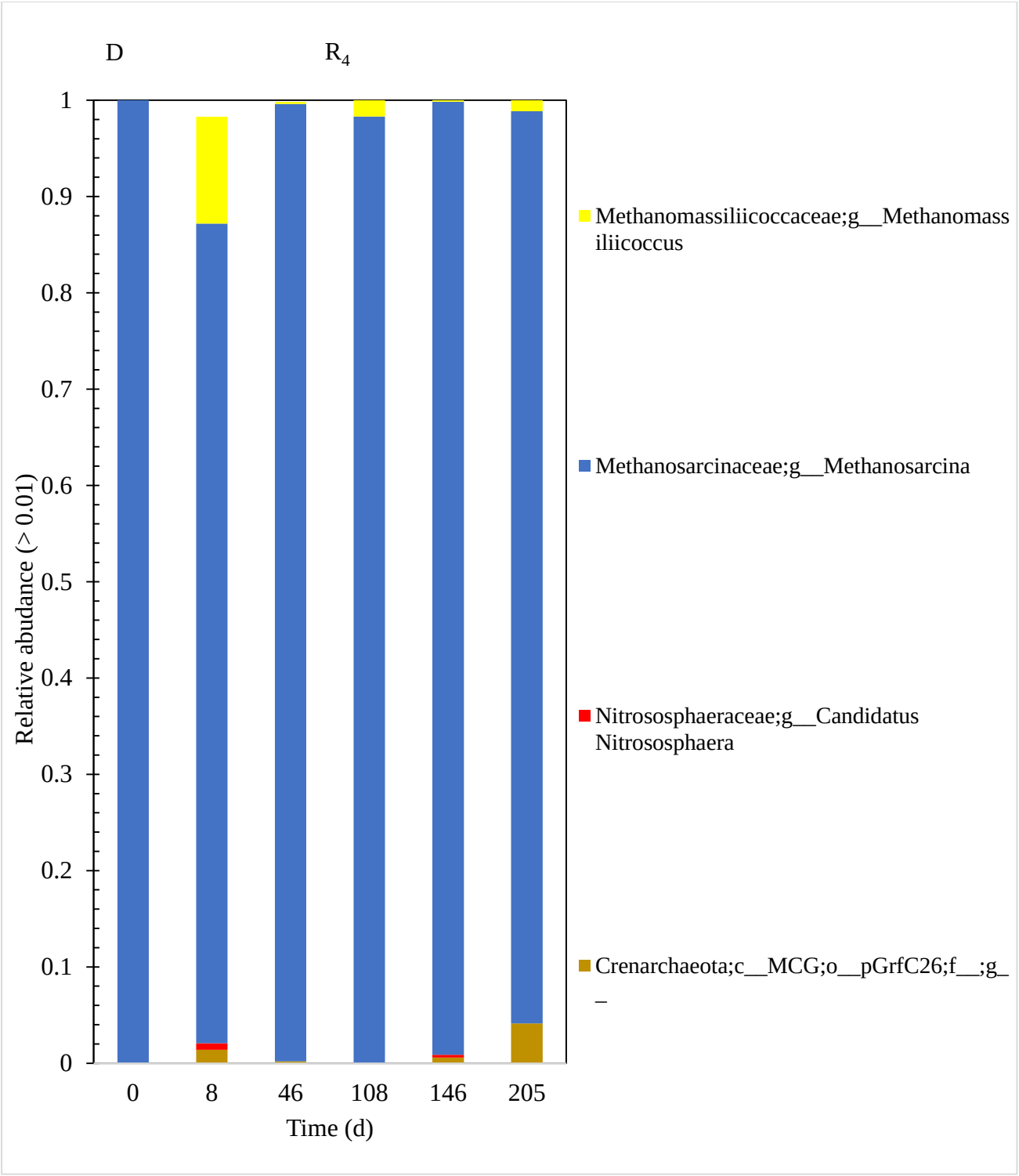


Figure 3-12: Composition of methanogens at the genus level for bioreactors. A and B: R_1 and R_2 respectively digesting steam-exploded wheat straw (SE). C and D: R_3 and R_4 digesting untreated wheat straw (WS)

3.6 Conclusion

In this study, the effect of a steam explosion pretreatment on the anaerobic biodegradability of WS was evaluated for the first time in a continuous feeding rate bioreactor system. Also, during the first part of this study, a screening BMP experiment was assessed to identify whether different steam explosion pretreatments, with severity's factor lying within the range 2.61 and 3.35, can enhance the biodegradability and the methane outcome from WS. The analytic measurements show an insignificant effect of the different pretreatments on the chemical composition of this residue while at the same time, the pretreatment seemed to affect mostly the structure. For the BMP experiment, all different steam explosion pretreatments increased the methane yields from WS while the quality of the raw material also affected the gas yields after the pretreatment. During the CSTR experiment, an average of 20% improvement on the methane production was offered by the pretreatment. Furthermore, the CSTR system proved to work under steady-state for long digestion periods and only mechanical issues limited the operation of the system. Finally, the results from this study revealed that the change of the OLR can affect the microbial populations of AD to a higher extent compared to the use of a steam explosion pretreatment. However, the system was able to adapt to the increased feeding rate without a significant decrease in biomethane production.

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4 Evaluation of steam explosion and mechanical pretreatment for the improvement of wheat straw biomethanation efficiency

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4.1 Abstract

Biomethanation of wheat straw (WS) represents a promising source of renewable energy. However, the rigid structure of WS results in insufficient digestion efficiency and low production yields. In this study, two different pretreatments (mechanical and steam explosion) were evaluated solely and in combination on WS before the final step of anaerobic digestion (AD). Experiments were conducted in a batch mode, as well as on a continuous mode with the use of a continuous stirring tank reactor (CSTR) system. Furthermore, the effect of the two pretreatments on the microbial population throughout the continuous experiment was also evaluated. According to the results from the BMP test, both pretreatments offered increased biomethane yields with additional improvement in biomethanation rate. The steam explosion contributed to improved gas yields while its combination with the mechanical pretreatment did not significantly improve the biomethanation further. In CSTR experiments, the four bioreactors were digesting steam-exploded straw (SE), steam-exploded and chopped straw (SEC), wheat straw (WS) and chopped wheat straw (WSC) respectively. Similar to the results from the BMP experiment, the steam explosion pretreatment achieved higher gas yields compared to the mechanical pretreatment. However, the two reactors digesting SE straw demonstrated also higher instability with fluctuations in the pH values, fast accumulation of intermediate products and low buffering capacity. The CSTR system demonstrated the ability to recover after the addition of a buffering solution and the adjustment of pH. The microbial populations inside the two bioreactors digesting steam-exploded straw were significantly different compared to the population found inside the two systems digesting either the untreated or the mechanical pretreated straw. On the other hand, the populations in the two reactors digesting steam-exploded straw (SE and SEC) did not statistically differ from each other. Similar results reported for

the two reactors digesting the two non-steam exploded substrates (WS and WSC). The above results indicate a higher effect of the steam explosion pretreatment, on the ecology of the AD, compared to the mechanical one.

4.2 Introduction

Several environmental problems, including climate change, are the result of excessive use of the planet's natural resources. The depletion of fossil fuels, together with the environmental problems that their use causes, increases the research interest towards alternative and renewable sources of energy. The idea of a circular economy is a modern approach for the economic growth of modern societies which aims at the creation of a sustainable environment and economic development. The main idea behind this strategy is to increase the lifetime of several products and materials and subsequently a decrease in the amount of the material that ends up in the landfills. At the same time, the production of renewable energy by the use of different organic waste comes in agreement with the idea of the circular economy (Abad et al., 2019). Anaerobic digestion (AD) is a well-examined biological process that is based on the biochemical interactions between members of a diverse and complex population of microorganisms for the production of renewable gas (biogas). Biogas can be utilised immediately towards the production of heat and electricity in combined heat and power (CHP) engines or it can be directly used as a transportation fuel (Haider et al., 2018). Biogas can be considered as an environmentally friendly biofuel, while its production is also a cost-effective way for reducing the organic footprint of different wastes, including lignocellulosic residues and several other organic wastes (Chandra et al., 2012a).

Lignocellulosic residues, such as wheat straw (WS), have been extensively studied as potential feedstocks in AD systems while they can also be considered as promising

alternatives to traditional feedstock such as maize (Cysneiros et al., 2012), corn silage (Ti et al., 2018) or food waste (Zarkadas et al., 2015). According to a previous study, WS represents the most abundant straw material in the UK, while its production equals 54% of the total production of straw, followed by oilseed rape (21%) and barley straw (20%) (w/w) (Copeland and Turley, 2008). Even though straw is typically used as feed for cattle, pigs, horses and beef, it has a low nutritional value when compared to oat or barley straw and it is primarily used as a bedding material for cattle, pigs and horses. However, significant amounts of this residue either remain unused in the fields or, even worse, are burned with a high impact on the environment and the air quality (Chandra et al., 2012b). According to Townsend et al., (2018), the production of WS in the UK is higher when compared to the demand. Due to its usually low anaerobic biodegradability, WS can be considered as a model substrate for AD systems representing challenging and slowly digestible feedstock. The low biodegradability of WS can be partially explained by its structure. The degradable fraction of WS mainly consists of the polymers cellulose and hemicellulose while lignin, which can be considered as non-degradable by the microorganisms of AD, also plays an important role in the composition of this residue. The high lignin content in WS combined with its cross-linking to relatively easily degradable polysaccharides also limits the efficiency of the AD process. Finally, the high crystallinity level of cellulose in WS is also a parameter that renders this type of biomass recalcitrant to biological and enzymatic attacks (Flint et al., 2012).

The efficiency of WS digestion can be improved by a pretreatment stage and/or by optimising the different vital operational parameters of the AD system including the hydraulic retention time (HRT), the organic loading rate (OLR) and the temperature. Various pretreatment methods have been proposed and examined in the past as an extra

step added before the AD of different lignocellulosic substrates. These can be categorised into four different groups: biological, chemical, mechanical and hydrothermal pretreatments. Chemical pretreatments are usually chosen as a method to modify or remove lignin and/or to reduce the crystallinity of cellulose (Kabir et al., 2014). Even though chemical pretreatment seems effective for the improvement of biogas production (X. Liu et al., 2015), its implementation usually requires an extra step towards the recovery of produced wastes. This increases the environmental concern and financial cost of the process. Biological pretreatments aim at partial biodegradation of the feedstock before the AD process with the use of fungi, bacteria or enzymes. Generally, biological pretreatments have been tested in the AD of lignocellulosic wastes with mixed results (Tsapekos et al., 2017b), while are also seen as expensive choices for commercialisation and are mostly used for research purposes. According to a recent study, the high cost of enzyme recycling is the main barrier for the use of this pretreatment in full-scale bioreactors systems (Hosseini Koupaie et al., 2019). On the other hand, both hydrothermal (e.g. steam explosion) and mechanical pretreatments (e.g. substrate grinding or chopping), do not require the addition of any harsh chemicals. In the steam explosion, only water is required for the pretreatment while this can be recovered from the AD process in agreement with the idea of a circular economy. Additionally, operational costs for both mechanical and hydrothermal processes are usually low.

Several mechanical pretreatments have been tested aiming to improve the anaerobic biodegradability of different types of lignocellulosic biomass. The particle size reduction of lignocellulosic biomass may facilitate greater access for AD microorganisms and enzymes to the biological tissues of the substrate (Motte et al., 2014). Moreover, reduction of cellulose crystallinity can also be achieved with the

application of mechanical pretreatment (Tsapekos et al., 2015), whereas a reduction in the lag phase of the AD system has also been connected to the mechanical pretreatment (Dahunsi, 2019). In continuous digestion systems, the mixing of the CSTR bioreactors and the management of the AD effluent are both easier when the particle size of the feedstock is decreased. However, more research is required to establish the optimal size reduction depending on the type of biomass and the AD operational conditions. For example, in a previous study, it has been reported that a decrease in particle size helped in biomethanation of lignocellulosic biomass, but excessive reduction can result in inhibition phenomena and decreased CH_4 yields (Kang et al., 2019).

In hydrothermal pretreatments, heat energy is used for a pre-set retention time, usually in combination with high pressure. During the steam explosion pretreatment, the increase in temperature and pressure inside the bioreactor is followed by a rapid depressurization which aims at breaking hydrogen bonds in the biomass structure. This disruption can potentially result in increased accessibility of the microorganisms of AD to carbohydrates in the feedstock (Tomás-pejó et al., 2017). In addition, after the steam explosion and depending on the severity factor of the pretreatment process (Theuretzbacher et al., 2015b), part of the lignocellulosic material is hydrolysed to simple sugars and passes to the liquid phase (Wang et al., 2018). This degradation of complex carbohydrates of WS can contribute to the enhancement of the biodegradation of the biomass during AD as well as to increased gas production.

Similar to mechanical pretreatment, the application of hydrothermal (steam explosion) pretreatment can also cause problems to an AD system fed with lignocellulosic residues. One of the disadvantages of this pretreatment method, especially when high temperatures are used, is the partial degradation of hemicellulose and the formation of aromatic compounds, such as furfural or 5-hydroxymethyl furfural (5-HMF), which can

potentially have an inhibition effect on the subsequent biogas production (Boopathy, 2009).

Apart from the pretreatment choice, an overall detailed design of the operational parameters of bioreactors is also required to ensure maximum biogas production and avoid inhibition problems. For that reason, even though steam explosion and mechanical pretreatments have been examined in the past (Menardo et al., 2012), a combination of the two pretreatments needs to be evaluated for their potential to increase the biomethane production of lignocellulosic residues.

This study aims to compare the effect of two different types of pretreatments and their combination, on the biomethanation process of WS. To this end, a BMP experiment was designed to test if the fractionation of biomass, and its combination with a steam explosion pretreatment, can offer an increased biomethanation for WS compared to the steam explosion pretreatment alone. On the second stage of this study, a four CSTR bioreactor system was used to digest four different feedstocks, including steam-exploded straw (SE), chopped steam-exploded straw (SEC), untreated wheat straw (WS) and chopped wheat straw (WSC) for 370 days. Over this period, the operation of the four bioreactors was monitored and changes were evaluated. Finally, periodical changes in the microbial composition inside the CSTR bioreactors were evaluated over the experimental period aiming to understand in-depth the effect of the two pretreatments on the biology of WS's AD.

4.3 Materials and methods

4.3.1 Pretreatment for BMP feedstock

The present study was conducted into two separated stages. In the first stage, a biomethane potential test (BMP) experiment was held examining the biomethanation of WS samples (control) or steam exploded WS samples after the application of a mechanical pretreatment (grinding). WS was collected from fields in the wider area of Northfolk, UK while part of this residue was steam-exploded (Economizer SE). The Economizer was fed with the raw WS and operated at a high temperature (155°C) and pressure (5 bars) for 14 min with a subsequent release of the generated pressure by a valve which caused a rapid depressurization inside the equipment. After the treatment, both SE and WS were transferred to the lab and were frozen (-20°C). Before the BMP experiment, both substrates were defrosted and dried in an oven at 80 °C for 48 h. Both WS and SE, after drying, were passed through a coffee grinder (De'Longhi, USA) to decrease their particle size. After grinding of samples, fractionation of the mechanically pretreated residues was attempted using different sized sieves (e.g. 1 cm, 0.2 cm, 0.1 cm and 0.05 cm) (Table 4- 1)

4.3.2 Preparation of BMP experiment

The effluent of four lab-scale CSTR anaerobic bioreactors was used as inoculum for BMPs. The working volume of the four reactors was 4 litres while one pair was fed with WS and the other with SE both at an OLR of 5 g VS/L day^{-1}. After collection, the two inocula were mixed and sieved to remove undigested particles of straw. The sieved inoculum was placed into plastic jars and incubated at mesophilic temperatures (42 $^{\circ}$C) for seven days aiming to minimise the endogenic CH_4 production. The inoculum was added in 150 mL glass vials along with the different fractions of WS and SE as feedstock. The organic loading rate for all BMPs was set at 9 gVS/L and the inoculum

to substrate ratio (I/S) at 3 (w/w). At the same time, blank trials (vials containing only the inoculum) were prepared in triplicate and incubated under the same conditions. The blank vials were used for measuring the endogenous CH_4 production while the obtained values were subtracted from those acquired from the vials with the substrate. Finally, positive control samples with microcrystalline cellulose (Avicel) were used to identify the efficiency of the inoculum as proposed before (Flores et al., 2015). After preparation, all BMP vials were flushed for 5 min with N_2 gas to ensure anaerobic conditions. BMP vials were manually shacked twice per day to minimise stratification problems and CH_4 production was measured daily. The produced gas was passing through a cylinder with a NaOH(aq) solution capturing CO_2 from biogas and through the water displacement method to measure only the desirable CH_4. NaOH(aq) solution was replaced by fresh after every three measurements. All BMP tests lasted 33 days and were carried out in triplicates alongside the positive controls and blanks. Finally, all gas measurements were reported and presented after correction to normal conditions (1 atm, 273 ^oK).

Table 4-1: The substrates used in BMP experiments comparing steam explosion and mechanical pretreatments of WS

Wheat straw (WS)	Steam exploded wheat straw (SE)
WS 1-0.2 cm ($WS_{1\text{-}0.2}$)	SE1-0.2 cm ($SE_{1\text{-}0.2}$)
WS 0.2 cm ($WS_{0.2}$)	SE 0.2 cm ($SE_{0.2}$)
WS 0.1 cm ($WS_{0.1}$)	SE 0.1 cm ($SE_{0.1}$)
WS 0.05 cm ($WS_{0.05}$)	SE 0.05 cm ($SE_{0.05}$)

4.3.3 Pretreatment for CSTR experiment

In the second stage of the present study, a system of four different CSTR reactors was used to examine the continuous digestion of raw and steam-exploded (SE) WS before and after the application of a mechanical pretreatment. The total experimental digestion period was 13 months. The initial feedstock used for this experiment (WS and SE) came from the same feedstock batches used during the first part of this study (BMP experiment). Both feedstocks arrived in the laboratory in 20 L plastic containers and were immediately frozen (-20 °C) until further use. For this experiment, chopping was used to reduce the particle size of the substrates, as it would allow the processing of larger quantities of WS for the reactors. To measure the effectiveness of the mechanical pretreatment, samples were collected and after drying at 105 °C overnight were passed through sieves for testing the particle size distribution. During the CSTR experimental period, the four bioreactors named R_1, R_2, R_3 and R_4 were digesting steam-exploded wheat straw (SE), steam-exploded and chopped wheat straw (SEC), untreated wheat straw (WS) and chopped wheat straw (WSC) respectively.

4.3.4 Set up and operation of the CSTR system

The CSTR system comprised four plastic bioreactors (R_1-R_4) with a total volume of 5 litres each, while their working volume was set at 3 litres. All digesters were used as a single biological replicate to digest the four different feedstock while the statistical repeatability of the results relies on the long digestion period for this experiment.

The effluent from the same four bioreactors which were operating under steady-state for the needs of another experiment examining (Chapter 3 of the present thesis) was used as inoculum. A total amount of 20 l coming from both AD effluents was mixed

and then used to re-inoculate the four bioreactors. All four different feedstocks (e.g. SE, SEC, WS and WSC) (Figure 4-1) were analysed every week for their total and volatile solids (TS, VS) (APHA., 2005). The outcomes from these measurements were used to calculate the daily feeding amounts of each feedstock. The organic loading rate (OLR) of the CSTR system was set at a level of 3 gVS/L day^{-1}, while the HRT was kept stable for the whole experimental period for 30 d. Aiming for a stable HRT, appropriate amounts of DI water were added daily in the bioreactors along with the feedstock. In addition, a commercially available mixture solution of nutrients necessary to the AD system was added daily in each reactor aiming to the improvement of the degradation efficiency, increased reactor stability and as a result increased biomethanation (Kainthola et al., 2019). After almost three HRT (90 days) the system demonstrated an instability with low pH values and thus it was decided the replacement the water added daily in the system with a solution (10 g/L) of sodium bicarbonate ($NaHCO_3$) as a method to balance the low buffering capacity of the system. After the addition of $NaHCO_3$, the system returned to a steady-state and operated for another three HRTs. Finally, for the whole experimental period, the daily addition of an appropriate amount of ammonium chloride (NH_4CL) was done to balance the high C/N ratio that WS was offering to the system.

Figure 4-1: The four materials used as feedstock to the continuous digestion system A: steam-exploded straw (SE) B: chopped steam-exploded straw (SEC) C: wheat straw (WS) D: chopped wheat straw (WSC)

4.3.5 Biogas composition

The composition of biogas in methane (CH_4) and carbon dioxide (CO_2) was weekly measured before feeding the reactors, with the use of a portable biogas analyser named BIOGAS 5000 (Geotech, USA).

4.3.6 Analytical techniques

All feedstock samples were examined in triplicate for total solids (TS) and volatile solids (VS) content (APHA., 2005). Dry matter content was calculated by drying the samples in a hot air oven at 105 °C overnight. After drying, samples were pre-ashed and ignited at 550 °C using a muffle furnace (Carbolite, Sheffield England) for at least

5 h. The volatile solids (VS) content was presented as the difference between the TS content and the produced ash (after the 550 °C drying process) divided by the weight of the wet mass. BMP feedstock was measured before the beginning of the experiment while for the CSTR feedstock, measurements were collected periodically to ensure the correct feeding rate. Furthermore, samples from both the BMP and the continuous experiment were also examined for their concentration in glucose, xylose and arabinose and lignin, including the acid-soluble and the acid-insoluble amounts, based on the protocol of Sluiter et al. (2012). Briefly, WS and SE were defrosted and dried at 40-50°C for a minimum of one day, then blended with the use of a coffee grinder. The reason for choosing a low-temperature drying was to avoid any chemical reactions which might occur in higher temperatures. In the next stage, 300 mg of each sample was weighted and 3 ml of concentrated sulfuric acid (72% w/v), was added to them. After that, all samples were placed in a water bath at 30 °C for 1 h, with brief vortexing every 10 min. Next, the concentration of sulfuric acid was diluted by the addition of 84 ml distilled (DI) water to a final concentration of 3.97 % of the total solvent (w/w). Subsequently, all samples were placed in a benchtop autoclave for 30 min at 125 °C (123 kPa). A duplicate of sugar recovery standard solution (SRS) was prepared and added in the autoclave along with the rest of the samples. In the next stage, samples including the two SRSs were removed from the autoclave and left to cool down at room temperature. After reaching 25°C, borosilicate Büchner filters (80 um) were used to separate the solid phase from the liquid phase. The solid fraction was dried at 105 °C overnight and ignited at 550 °C for at least four hours to measure acid-insoluble lignin and ash. In addition to the above procedure, the liquid fraction was collected and used to determine the acid-soluble fraction of lignin in the initial samples. For the needs of these measurements, spectrophotometry (at 320 nm) was used after the appropriate

dilutions according to the instructions of Sluiter et al., (2012). Finally, 20 ml of the remaining liquid fraction were neutralized to pH 5-6 with the use of $CaCO_3$, pass through a polypropylene filter with a pore size of 0.2 μm and further examined at the HPLC system.

4.3.7 HPLC system

The HPLC system used in this study was an Agilent 1260 series system. The system was coupled with an Aminex HPX-87H column (Biorad) with a length of 300 mm and a diameter of 7.8 mm. The temperature of the HPLC column was set at 65 °C and the mobile phase was 5 mM H_2SO_4 with a flow rate of 0.6 mL/min. The sample's volume for every injection was set at 20 μL. The HPLC system was using a diode array detector (DAD) and a refractive index detector (RID) connected in series. Furthermore, external calibration curves of the sugars xylose, arabinose and glucose, the VFAs (acetic, propionic, butyric, isobutyric, valeric and isovaleric acid) as well as furfurals and 5-HMF were prepared using commercial samples as standards.

4.3.8 DNA extraction and sequencing analysis

Changes in the microbial population over the CSTR experimental period were monitored in samples collected at the beginning and the end of the experiment, before and after two short periods of reactor's failure and at periods of reactor's steady-state operation. It was also decided to include one sample from a period when the accumulation of VFA inside the reactor was high, the pH was low and the biogas production was below the average production compared to the rest of the experimental period (day number 210). Triplicate samples of 0.25 g from selected reactor's effluents were isolated and processed according to the instructions of the isolation kit QIAamp® RowerFecal® DNA Kit (QIAGEN, Germany) protocol for the isolation of DNA from

gut material, stool samples and biosolids. The concentration of DNA on the isolated samples was measured with the use of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Rockland, DE). After that, all samples were sent to an external laboratory (Novogene, Hong Kong) to run the microbial amplicon-based metagenomics sequencing (Illumina PE250, Q30 ≥75%). Total genome DNA from samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. According to its concentration, DNA was diluted to 1ng/μL using sterile water. The Amplicon Generation was conducted using 16S rRNA/18SrRNA/ITS genes of distinct regions (16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified used specific forward primer 341F (CCTAYGGGRBGCASCAG) and a reverse 806R (GGACTACNNGGGTATCTAAT). For the Archaeal, 16S DNA the hypervariable regions V4-V5 (397bp) were amplified using the primers Arch519F (CAGCCGCCGCGGTAA) and Arch915R (GTGCTCCCCCGCCAATTCCT). All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Subsequently, a mixture of equal volumes of 1X loading buffer (containing SYB green) with PCR products was prepared and electrophoresis on 2% agarose gel was run for detection. Samples with a bright main strip between 400 and 450 bp were chosen for further evaluation. Subsequently, PCR products were mixed in equal density ratios and then, the mixture of the PCR products was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using NEBNex t ® Ultra DNA Library Prep Kit for Illumina, following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina platform and 250 bp paired-end reads

were generated. Regarding the interpretation of the results, all microorganisms found to have a relevant abundance below 1% of the total population were disregarded as proposed before (Strang et al., 2017).

4.4 Results and discussion

4.4.1 Chemical composition of the feedstock

The chemical composition of WS and steam-exploded straw before and after the application of mechanical pretreatment (gridding) was evaluated. A statistically insignificant decrease in the hemicellulose (xylose and arabinose) content was noted after the application of both the steam explosion and mechanical pretreatment (Fig. 4-2). More specifically, the hemicellulose content in WS was equal to 37% of the total biomass (w/w) while in SE at the smallest particle size ($SE_{0.05}$) was lower than 30%. This decrease is considered low compared to the decrease in hemicellulose concentration after SE pretreatment reported in previous studies when a reduction of up to 40% was found after a 220 °C for 6 min treatment of WS (Cui et al., 2012). This can be attributed to the low severity factor of the steam explosion equipment used in the present study. One of the parameters that affect the severity factor is the pretreatment temperature (Iroba et al., 2014). In the present study, a temperature of 155°C was chosen for the steam explosion process while Cui et al. (2012) worked at a temperature range between 200 and 220 °C. Studies related to the hydrothermal pretreatment of lignocellulosic residues have found that parameters other than temperature, including the pretreatment retention time, can potentially affect the composition of the under pretreatment material (Garrote et al., 1999). As a result, the short retention time used in the present study might have also played a role in limiting the hydrolysis of hemicellulose. Furthermore, the effect of the two pretreatments was also not clear on

the percentages of cellulose and total lignin (AIL and ASL), while the concentration of these two components did not follow a specific pattern for the different pretreated samples. More spesificaly, cellulose content was 47% (w/w) in the untreated WS while it ranged between 40% and 49% (w/w) for the rest of the samples. The percentage of lignin was between 17% and 20% (w/w) for all examined feedstock. The values for WS are in agreement with previous reports on WS (Bolado-Rodríguez et al., 2016). Furthermore, similar composition values of lignin and cellulose in WS samples has been reported after a combination of mechanical and SE pretreatments (Theuretzbacher et al., 2015a).

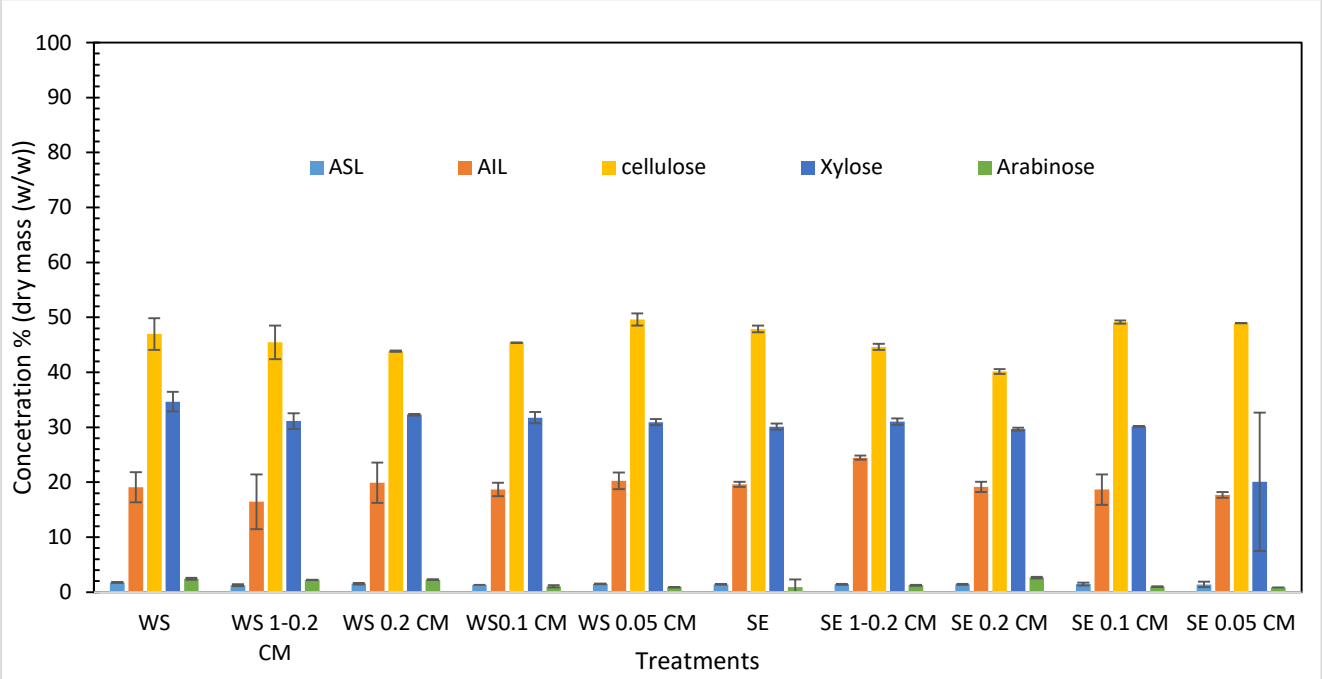


Figure 4-2: Chemical composition of wheat straw (WS) and steam-exploded straw (SE) before and after the mechanical pretreatment. * ASL: Acid Soluble Lignin; AIL: Acid Insoluble Lignin

4.4.2 Distribution of CSTR's feedstock's particle size

In the BMP experiment, the different particle sizes were separated and digested separately as seen in table 4-1. On the other hand, in the CSTR experiment, no further separation after the mechanical pretreatment was done while particle size was measured only for research purposes. After passing through different sizes of sieves the different fractions of the four CSTR feedstock were separated based on their particle size as seen in Fig 4-3 into four different categories (bigger than 0.2 cm, 0.2 cm, 0.1 cm and 0.05cm). According to the results from these measurements, the mechanical pretreatment (chopping) affected on a higher extend the particle size of WS compared to the steam explosion which has previously been shown to reduce the particle size of lignocellulosic residues (Tang et al., 2018). As seen in Fig. 4-3, the chopping of WS significantly reduced the particle size from the initial size to smaller to 1 cm. On the other hand, when the same pretreatment applied to the SE, the effects were not as clear since the distribution of the different particles sizes was similar before and after the application of the mechanical pretreatment.

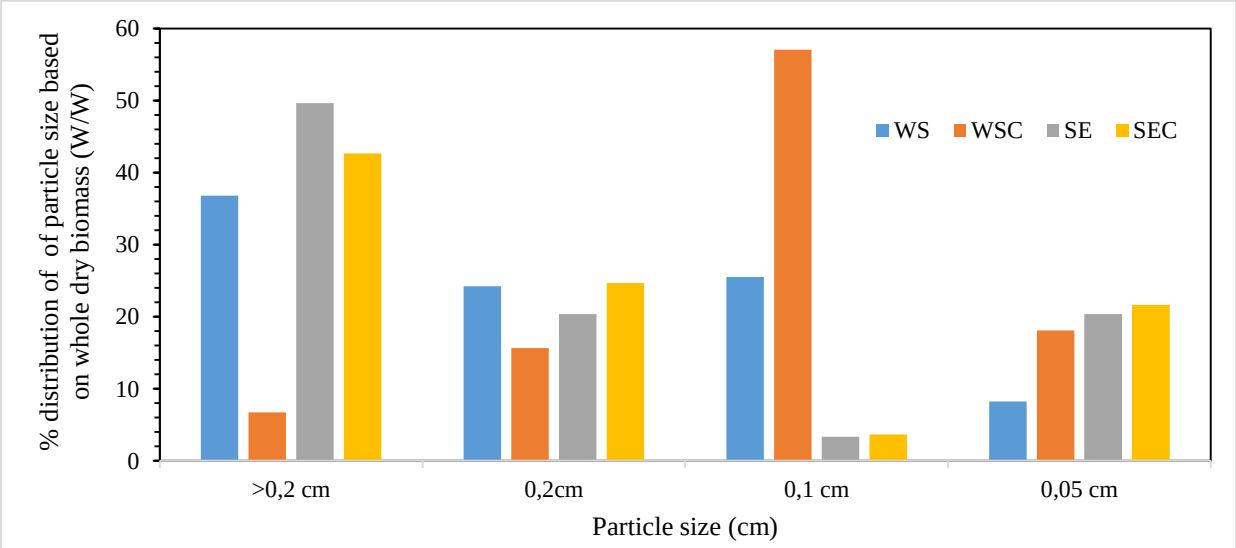


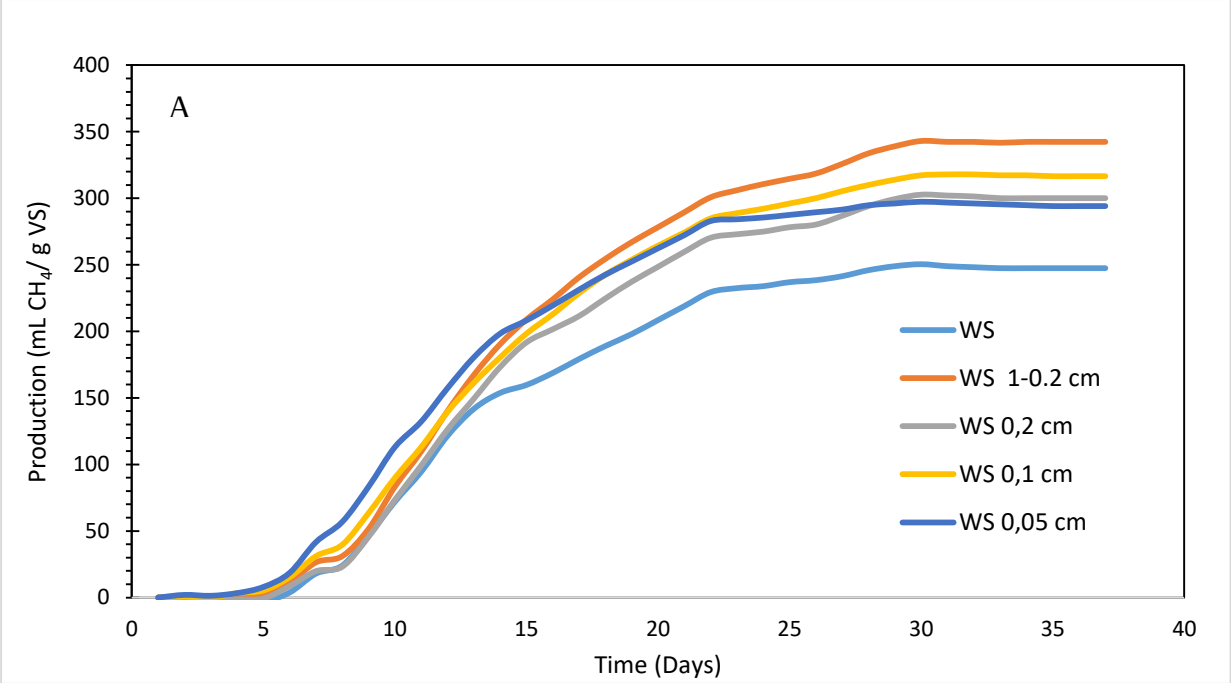
Figure 4-3: The percentage distribution of each particle size against the whole amount of the dry biomass

4.4.3 BMP digestion of steam-exploded and/or mechanically pretreated WS

The main aim of the BMP experiment was to evaluate the effect of two different pretreatment methods on the anaerobic biodegradability of WS. Steam explosion (hydrothermal) and grinding (mechanical) were the two pretreatments chosen to be evaluated. Furthermore, both pretreatments were combined aiming to test any further improvement on the biomethanation of WS. Both steam explosion and mechanical pretreatment had a positive effect on the biomethane production from WS (Figure 4-3 a, b). The untreated WS demonstrated a biomethane production close to 250 ml CH_4/g VS_{added} while the production from the steam-exploded straw samples reached values up to 335 ml CH_4/g VS_{added} (equivalent to 34 % (v/v) improvement). At the same time, the mechanical pretreatment offered increased biomethanation rates with the final accumulation of gas ranging from 294 to 342 ml CH_4/g VS for the different fractions of WS used (Fig. 2a). The highest CH_4 production was reported for the $WS_{1\text{-}0.2}$ while the production seemed to decrease slightly when the smaller particle size substrate was used. Studies have reported the existence of a threshold for the decrease in particle size of WS before anaerobic digestion. Dumas et al., (2015) reported this threshold as 0.02 cm. According to the same study, a higher decrease of the particle size would not change further the crystallinity index of the feedstock and along with the rapid production of VFAs can potentially cause inhibition to the system if the inoculum used is not acclimatized to similar conditions. However, alternations in equipment used for grinding feedstock, differences in BMPs preparation and possible feedstock composition variations, can potentially explain differences between the above study and the results from the present study.

Furthermore, the combination of mechanical and steam explosion pretreatments was also evaluated. According to the results, the biomethane production was not

significantly improved in any particle size fractions of the steam-exploded straw (Fig. 2b). However, even though the increase in the biomethane production was not significant, the $SE_{0.2}$ produced higher gas yields (370 ml CH_4/g VS) compared to all other fractions. The accessibility of the AD microorganisms on degradable straw components seems to be already increased after the steam explosion pretreatment and this is probably the reason for the low increase in the produced yields when the two pretreatments were combined. The comparison between the two pretreatments shows that the steam explosion pretreatment had an effect on the deconstruction of WS's structure and probably this is another reason why the additional gridding step was not as efficient as in the case of the WS digestion.



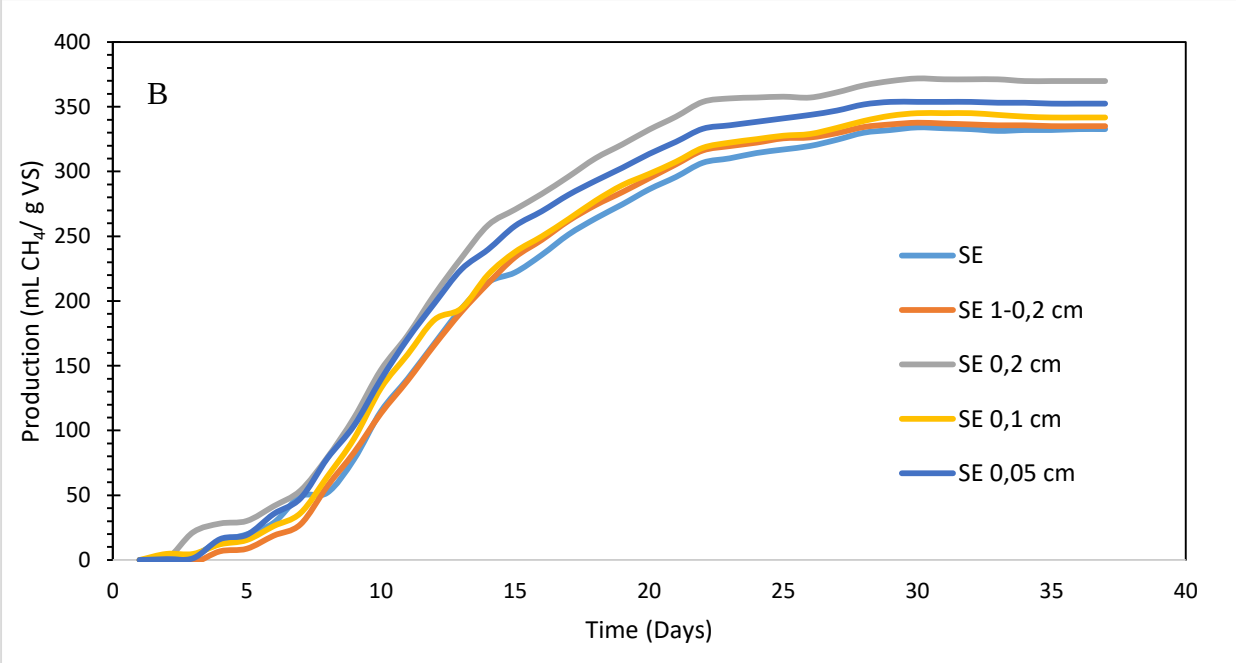


Figure 4-4 (a, b): Daily accumulation of CH_4 produced from the mechanical pretreated WS samples (a) and the mechanical pretreated SE samples (b)

4.4.4 The CSTR digestion of steam-exploded and/or mechanically pretreated WS

A CSTR system was used to evaluate steam explosion, chopping and their combination for anaerobic biodegradability of WS on a continuous mode. The daily biogas production from the four bioreactors was assessed and while whole experimental period was separated into 3 different sub-periods (A), (B) and (C) according to the performance of the reactors. During period (A) bioreactors' performance was relatively stable with a small period between days 70 and 78 when a high decrease was reported on the produced biogas yields in all of them. During this period, the pH of the reactors dropped dramatically and even reached values close to pH 5, which is well below the optimal range for AD systems. For the same period, VFAs accumulation was also increased. After a partial re-inoculation (10% v/v) of all bioreactors with effluent which was weekly removed from the same system throughout the experiment, and the addition of 10 g/L sodium bicarbonate, the system recovered and operated at a steady-state for another 2.5 HRTs (75 d). During this period (A), WS demonstrated the lowest biogas production followed by the chopped WS (WSC). At the same time, the steam-exploded and chopped WS (SEC) seemed to be superior to the SE with regards to biogas production yields offering on an average close to 10% (v/v) increased gas yields. During sub-period (B) the whole system was unstable accompanied by an acidic pH (Figure 4-6) and high accumulation of VFAs (Figure 4-5). Also, the percentage of the produced biogas in CH_4 was dropped, especially for the steam-exploded straw reactors (Figure 4-7 (a- d)). In an attempt to recover the system, on day 263 the water added daily to the system was replaced with a 10 g/L sodium bicarbonate ($NaHCO_3$) aqueous solution. After a short recovery period, the biogas production, especially of the two reactors fed with steam-exploded straw, seemed to be very high, probably due to the

reaction of sodium bicarbonate with accumulated acids in the system and the production of CO_2. After this short period, the system returned to steady-state and remained relatively stable for a period of three HRTs (90 days) which is indicated as sub-period (C) (Figure 4-4). The most unstable reactor, but also the most productive regarding biogas production during steady-state, was the one fed with SEC. Furthermore, the same reactor was the one that demonstrated the highest fluctuations in the pH and the presence of VFAs (Figure 4-5 and 4-6). Worth mentioning is the fact that gas production from the WSC reactor was superior to that of the WS reactor for most of the experimental period (an exception was reported during the period (C) where the results were reversed). This cannot be attributed to the accumulation of VFAs in the WSC reactor as proposed in a previously published study (Dumas et al., 2015), as the accumulation of the acids was below the HPLC detection limit after day 100 (Figure 4-5). A partial depletion of some micronutrient in the reactor digesting WSC could potentially explain the decrease in the produced yields for this specific bioreactor.

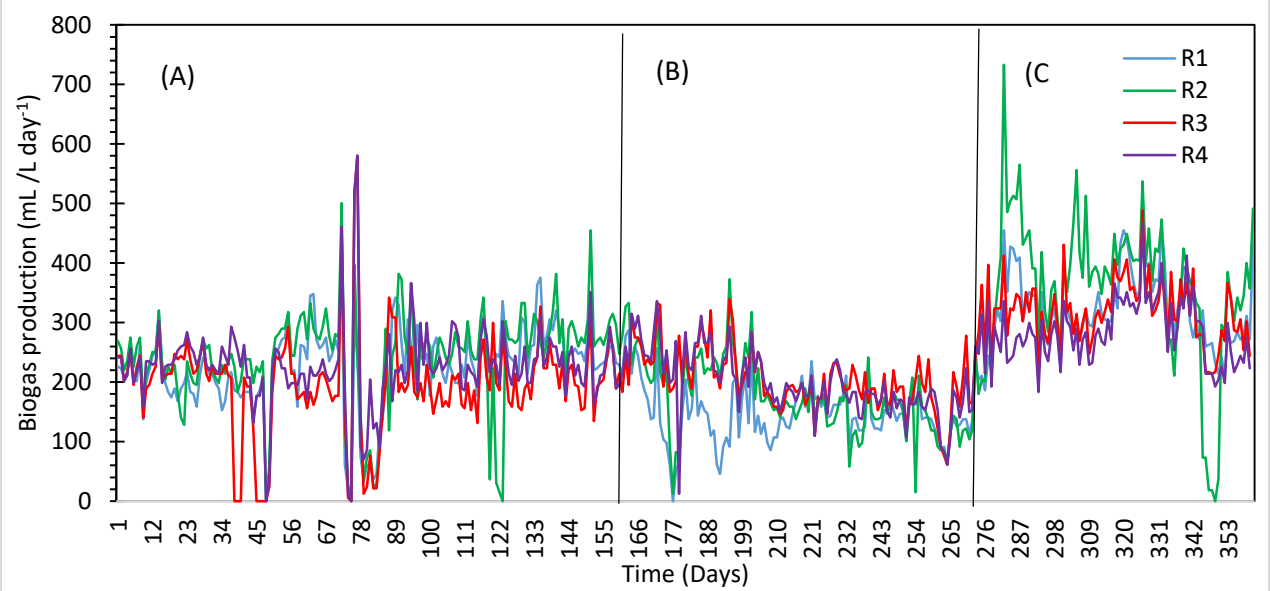


Figure 4-5: Daily biogas production for the reactors R_1: Steam exploded straw (SE), R_2: steam exploded and chopped straw (SEC), R_3: wheat straw (WS) and R_4: chopped wheat straw (WSC)

4.4.5 VFAs profile

The high fluctuations of biogas yields during all three different sub-periods can be partially attributed to the high accumulation of the total VFAs (Figure 4-5). It is also worth mentioning that the accumulation of the VFAs was more profound for the two bioreactors digesting steam-exploded straw. At the same time, for the WS and WSC digesters, the presence of acids was usually below the HPLC detection limit with an exception for days 84 and 94. During the first relatively stable period (A), except for one short period between days 77 and 94, biogas production was similar to the yields reported for WS in previous studies (Shi et al., 2017). The VFA profile from that period can explain the daily biogas production while the relatively stable gas yields were decreased only when the presence of the VFAs was increased in the system (days 77 to 94). Similarly, for the period (B) where biogas production was continuously decreasing, the VFA accumulation followed the opposite trend and increased to levels higher than 10 g/L for the total VFA. The two VFAs that were found predominantly were acetic and propionic followed by butyric acid. Similar VFAs trends were described in previous work were mechanical pretreatment of WS was used before the AD process (Dumas et al., 2015). The partial re-inoculation of 10% of the initial working volume of the reactor, followed by the daily addition of sodium bicarbonate helped the system to recover relatively fast. Finally, a progressive decrease in the concentration of the total VFAs was also observed during period (C) when the buffer solution ($NaHCO_3$) was added daily in the system.

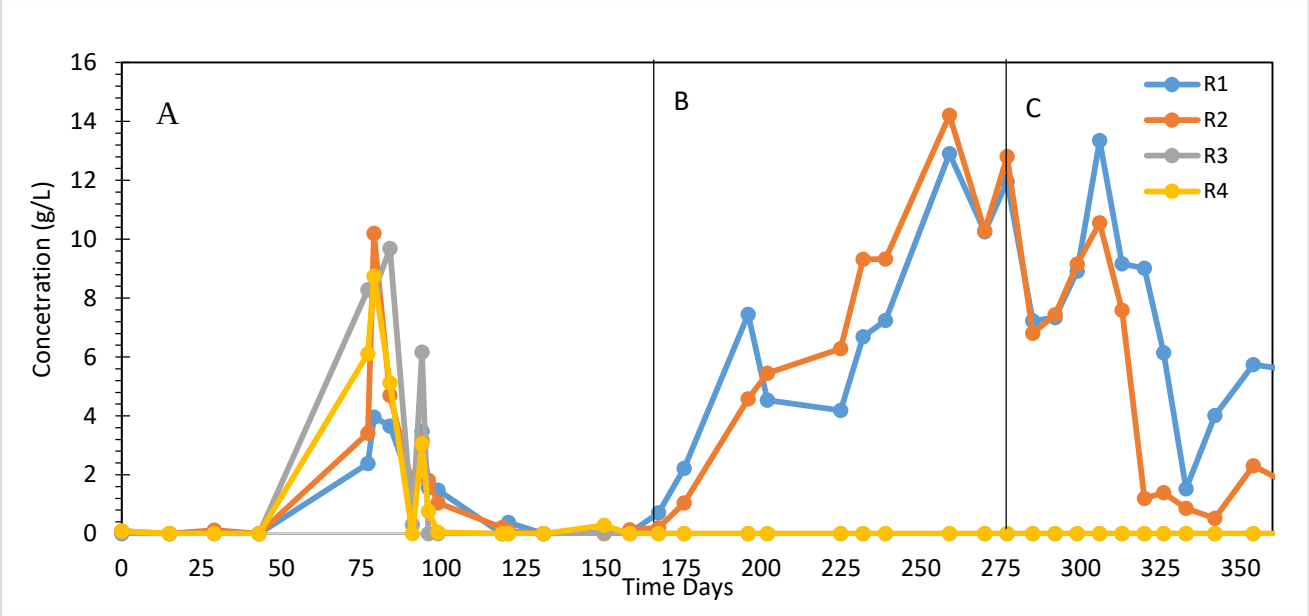


Figure 4-6: Total VFAs profile for the four bioreactors though out the experimental period. R1: Steam exploded straw (SE), R2: steam exploded and chopped straw (SEC), R3: wheat straw (WS) and R4: chopped wheat straw (WSC)

During the CSTR experiment, the bioreactors that were digesting steam-exploded straw seemed to have lower buffering capacity with higher sensitivity to pH changes compared with those fed with WS (R_3, R_4) (Figure 4-5). Similarly, low buffering capacity phenomena have been reported for AD system fed with lignocellulosic residues (Nkemka and Murto, 2013). Measurements of the buffering capacity can be used as a method to predict the reactor's unstable performance (Ward et al., 2008). According to Ward et al. (2008), high accumulation of VFAs can reduce the buffering capacity before the drop of the pH (Ward et al., 2008). However, the pH represents an important parameter for AD systems while changes in this can cause a decrease in specific microbial populations inside the bioreactor. As an example, in a previous investigation, the population of methanogenic Achaeabacteria was greatly reduced when the pH of the system fell below 6.6 (Ward et al., 2008).

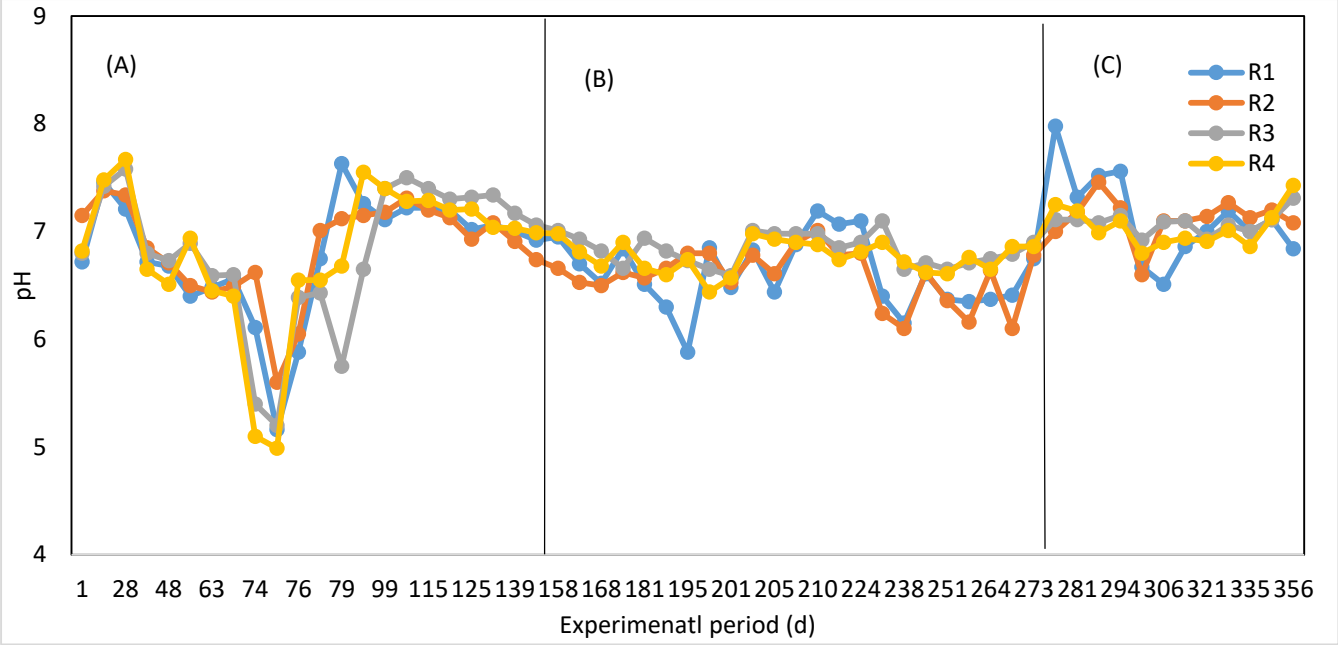


Figure 4-7: The pH of the four bioreactors over the whole CSTR experimental period. R1: Steam exploded straw (SE), R2: steam exploded and chopped straw (SEC), R3: wheat straw (WS) and R4: chopped wheat straw (WSC)

Total and volatile solids (TS, VS) can also be used as an indicator for the efficiency of the anaerobic digestion system mostly due to mass transfer limitations according to Abbassi-Guendouz et al. (2012). According to Figure 4-6 (A, B), the digestion of the untreated and mechanical pretreated straw WS (WS, WSC) was more stable compared to the digestion of the two steam-exploded straw feedstock (SE, SEC). Moreover, the mechanical pretreatment led to a decrease in solids content inside the digester fed with SEC compared to reactor digesting SE, in which steam explosion pretreated straw was exclusively used as feedstock. It seems that in terms of the reduction of solid content in the reactors, the mechanical pretreatment worked better than the steam explosion. These results might have an important value, especially for industrial-scale systems since a decrease in the solids content of the bioreactor can be associated with the easier operation of the system by reducing some mechanical issues caused by the digestion of untreated straw or other lignocellulosic residues (Kang et al., 2019).

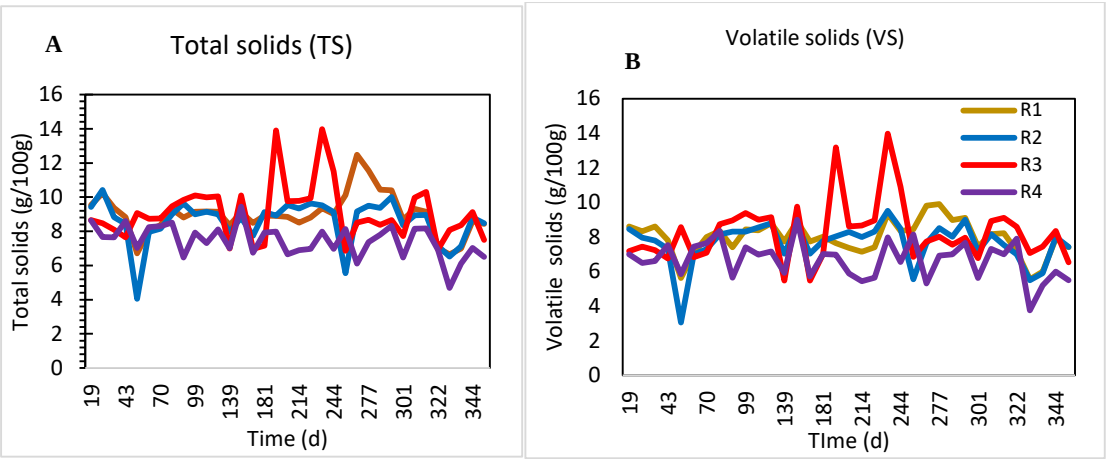


Figure 4-8: The total (TS) (A) and volatile (VS) (B) solids content on the effluent of the four bioreactors over the whole CSTR experimental period. R_1 was digesting steam-exploded wheat straw (SEC), (B) R_2 was digesting steam-exploded and chopped wheat straw (SEC), (C) R_3 was digesting untreated wheat straw (WS) and (D) R4 was digesting chopped wheat straw (WSC)

The operation stability of the four bioreactors affected the concentration of the produced biogas in CH_4 and CO_2. The mechanical pretreatment seemed to affect much less the stability of the system compared to the steam explosion pretreatment. As demonstrated in Figure 4-8 (A-D), the produced CH_4 in bioreactors R_3 and R_4, where non-steam exploded straw was digested, was relatively stable at a percentage of 50% of the total biogas yield. On the other hand, in both digesters operated with steam-exploded straw (SE, SEC), a fluctuation between the production of CH_4 and CO_2 was noted. The instability of the pH values on the above-mentioned bioreactors followed by the addition of sodium bicarbonate resulted in an increase in the percentage of CO_2 compared to CH_4. The CH_4 content was very low in both reactors for the period between the days 175 and 210, however, the system seemed to recover after the addition of sodium bicarbonate while the CH_4 production reached values similar to those reported previously in the literature (Babaee and Shayegan, 2011). These are normally between 50–70% and 30–50% for CH_4 and CO_2 content in biogas respectively. However, the

composition of biogas can fluctuate during the operation of an AD system due to changes in the pH (Angelidaki et al., 2018).

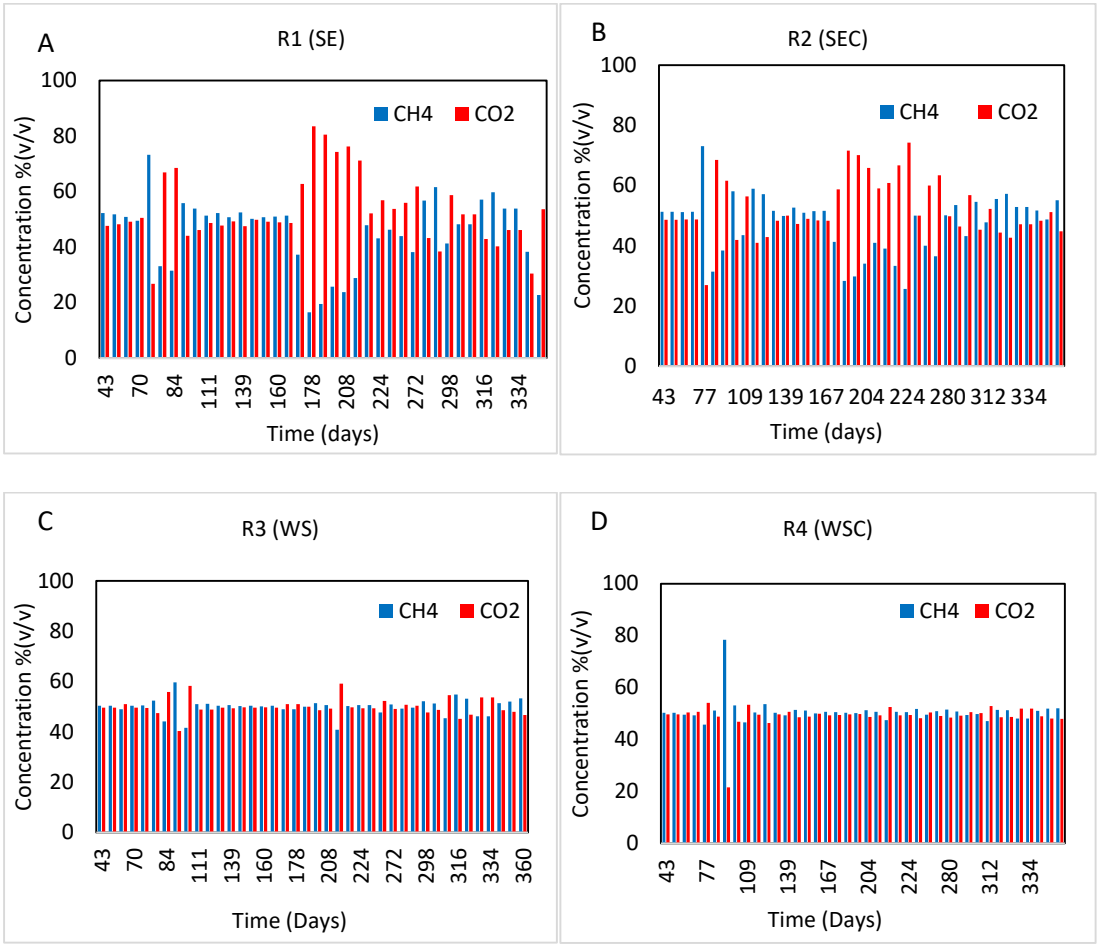


Figure 4-9 (A-D): The % composition of biogas to CH_4 and CO_2 for (A) R_1 digesting steam-exploded wheat straw, (B) R_2 digesting steam-exploded and chopped wheat straw, (C) R_3 digesting wheat straw and (D) R_4 digesting chopped wheat straw for the whole experimental period

4.4.6 Bacterial composition in the CSTR system

The microbial communities of the four bioreactors digesting SE, SEC, WS and WSC respectively were also examined throughout the CSTR experimental period. Also, in order to ensure the statistical significance of these analyses, considering also the fact that each bioreactor was digesting a different feedstock, three replicates were collected from each bioreactor and for each chosen time point. The purpose of these analyses was to evaluate the effect of the two pretreatments in the microbiology of the system.

According to the microbial analysis, the two bioreactors digesting the non-steam-exploded straw (R_3, R_4) have a significantly higher Shannon diversity index (H) compare to the digesters fed with either SE or SEC (R_1 and R_2). An increased H-index suggests higher biodiversity and as such, it could be assumed that steam explosion pretreatment resulted in decreased complexity and richness of microbial populations in the bioreactors. This decrease in the biodiversity of the AD system might also responsible for the instability of those two reactors. According to a recent study, the type of substrate is a key factor, affecting the composition of a microbial population during AD, more than the initial composition of the inoculum (Poszytek et al., 2017). Additionally, when ensiled energy crop was co-digested with barley straw, it was found that microbial communities were affected significantly by the added amounts of straw (Feng et al., 2020). Another interesting outcome was the fact that the mechanical pretreatment seemed to negatively affect the microbial population when WS was digested but not in the case of SE digestion. The increase in the microbial richness for the reactor digesting chopped wheat straw (R_4) after day 250 can be attributed to the partial re-inoculation applied to aid the system to recover the increased presence of VFAs and the low pH. As a result, clear conclusions cannot be made regarding the significance of the effect of mechanical pretreatment on the microbial population of AD (Figure 4-9).

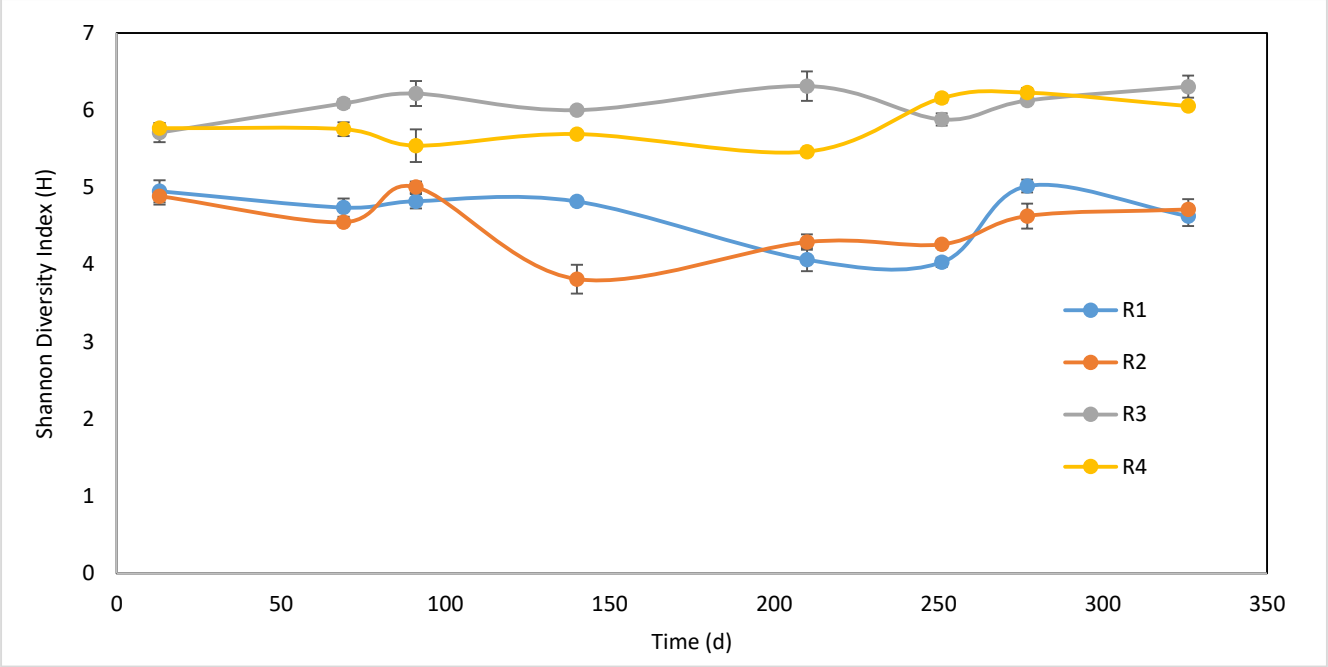


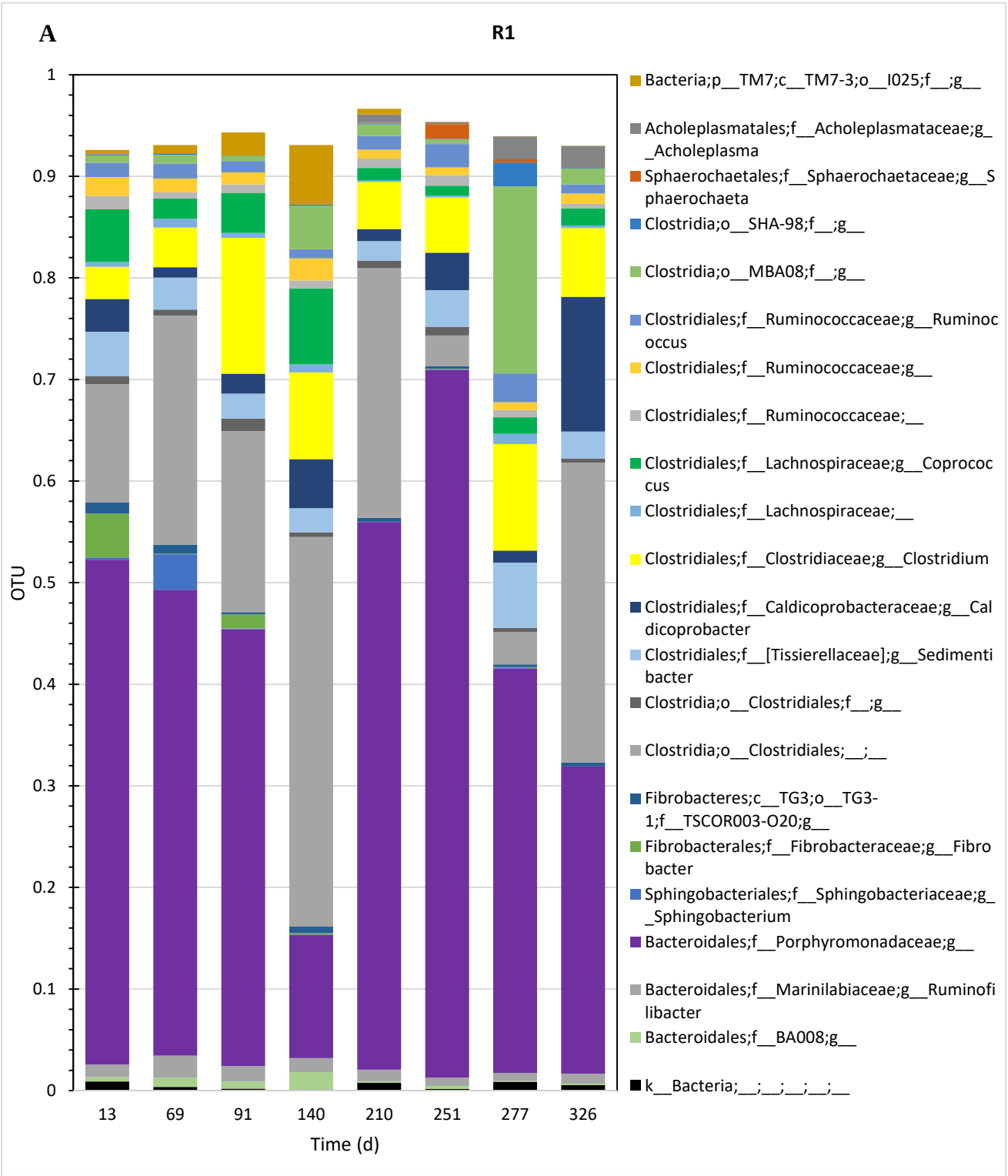
Figure 4-10: Shannon diversity index (H) for bioreactors digesting steam-exploded wheat straw SE (R_1), steam-exploded and chopped wheat straw SEC (R_2), wheat straw WS (R_3-control) and chopped wheat straw WSC (R_4)

Apart from differences in their richness, the reactors digesting steam-exploded straw (R_1 and R_2) had important differences in presence of specific species, compared to reactors digesting WS and WSC (R_3 and R_4). In more detail, in the two reactors digesting steam-exploded straw (R_1 and R_2) the most abundant bacteria families found were *Porphyromonadaceae*, *Clostridiales*, *Clostridiaceae* and *Caldicoprobacteraceae* while in R_2 an unidentified type of bacteria (MBA08;f__;g__) was also present, up to 10%, during the last two days of the experiment. The microorganisms that belong to the family of *Porphyromonadaceae* are associated with the production of acetic acid from the utilisation of carbohydrates and proteins (Dong et al., 2016) while this can explain the importance of this microbial family in the AD of lignocellulosic residues. Generally, during AD, the produced acetic acid is subsequently utilised by the acidegonotrophic methanogens towards CO_2 and CH_4 production. The presence of *Porphyromonadaceae* in the digesters fed with SE and SEC was found at numbers up

to 70% of the total population which was significantly higher compared to the presence of this family on the digesters fed with either WS or WSC. More specifically, for R_3 (fed with WS) this family accounted for 23% only on day 13 while in R_4 (fed with WSC) the same microorganism accounted for 43 % on day 91 and then decreased to levels below 12% until the end of the experiment. These microorganisms have previously been reported as the most dominant family in processes where easily degradable lignocellulosic feedstock, such as maize silage, was digested (Lv et al., 2019). As proven in the present study, both steam explosion and mechanical pretreatment render the digestion of WS easier since they can potentially break down the recalcitrant structure of this residue. As a consequence, the increased presence of the *Porphyromonadaceae* family can be attributed to these alternations caused by the pretreatments. In addition, an unidentified species from the family of *Clostridiales* (*Clostridiales;__;__)*, was the second most abundant family found in reactors fed with SE and SEC but at the same time was only found on up to 10% of the total microbial population in the systems fed with either WS or WSC. Members of this family were previously found to play an important role in the degradation of lignocellulose (Strang et al., 2017). Next, the family of *Clostridiaceae,* including the genus *Clostridium* sp., was counted for all four bioreactors to range between 3-18% (w/w) of the total microbial population. Members of this family were used in a previous study as a biological additive (bio-augmentation factor) in the digestion of oil palm empty fruit bunches (EFB) (Suksong et al., 2019). Finally, the fourth most abundant family in both bioreactors digesting steam-exploded samples (R_1 and R_2) was *Caldicoprobacteraceae* and more specifically the genus *Caldicoprobacter* sp. which is also known for its ability to degrade proteins and hemicellulose according to Bouacem et al., (2015). On the other

hand, *Caldicoprobacter* sp. was only found close to the threshold of 1% for both R_3 and R_4.

In contrast to R_1 and R_2 (SE and SEC) where *Porphyromonadaceae* was by far the most abundant family counted, in R_3 and R_4 (WS and WSC), no microbial family found in numbers significantly higher than the rest of the families (Figure 4-10 C, D). However, an unspecified clostridia species (*Clostridia;o__MBA08;f__;g__*) was found to increase its numbers over time, with the highest levels to be found for R_3 on day number 210 (36%), as seen in (Figure 4-10 C). Furthermore, the *Lachnospiraceae* family (genus *Coprococcus* sp.) was found in percentages within a range of 3-20% (w/w) and 5-15% for R_3 and R_4 respectively while in R_1 and R_2 it was found on slightly lower concentrations (1-10%). Finally, an increase in the abundance of the family *Marinilabiaceae* (genus *Ruminofilibacter* sp.) up to 20% inside R_3 was reported after day number 13 while for R_4 the presence of this microorganism was high only on days 251 and 277 (17 and 15% respectively).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Clostridia;o__MBA08;f__;g__
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Sphingobacteriales;f__Sphingobacteriaceae;g__Sphingobacterium
Bacteroidales;f__Porphyromonadaceae;g__
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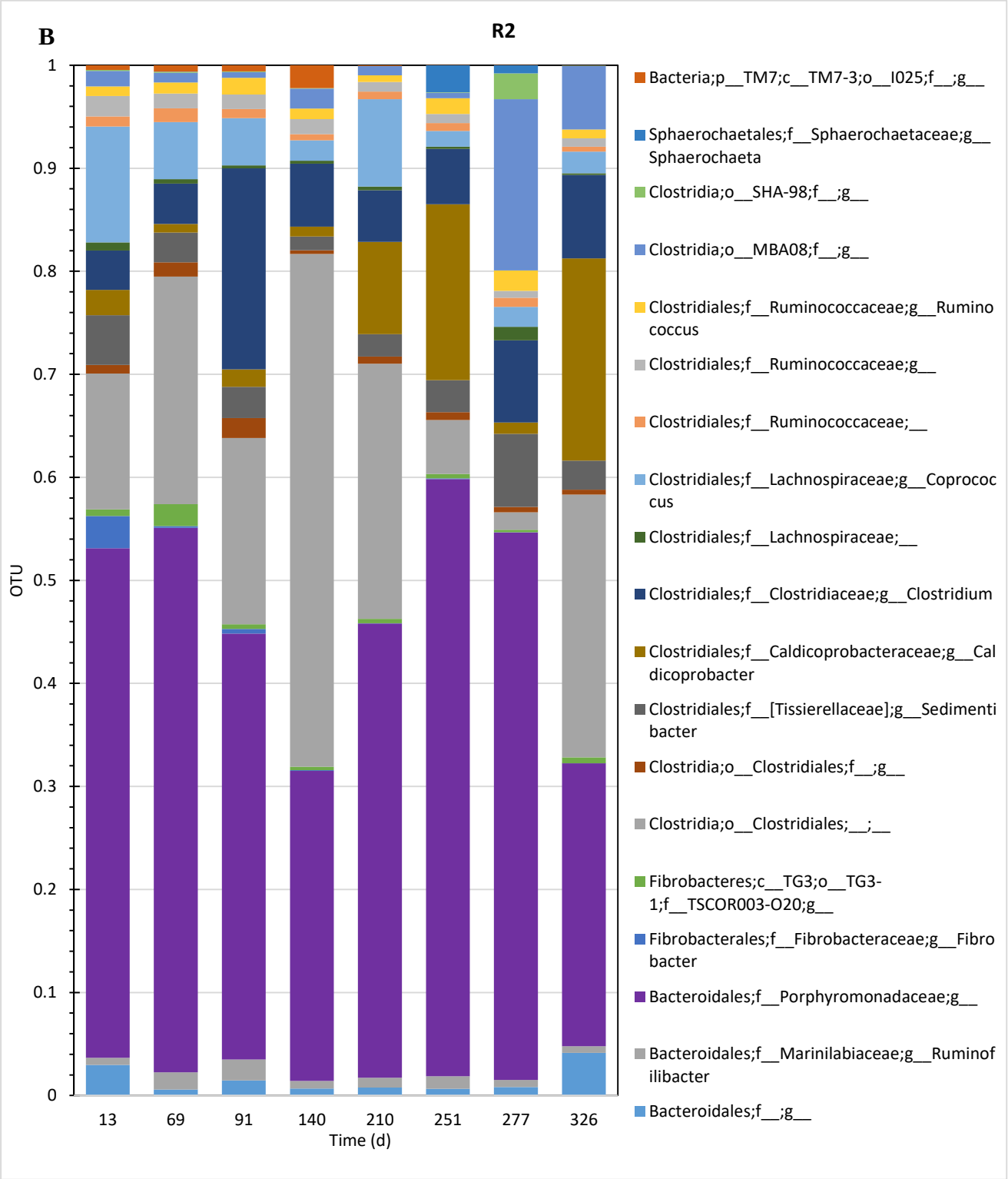
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Clostridia;o__SHA-98;f__;g__
Clostridia;o__MBA08;f__;g__
Clostridiales;f__Ruminococcaceae;g__Ruminococcus
Clostridiales;f__Ruminococcaceae;g__
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Clostridiales;f__[Tissierellaceae];g__Sedimentibacter
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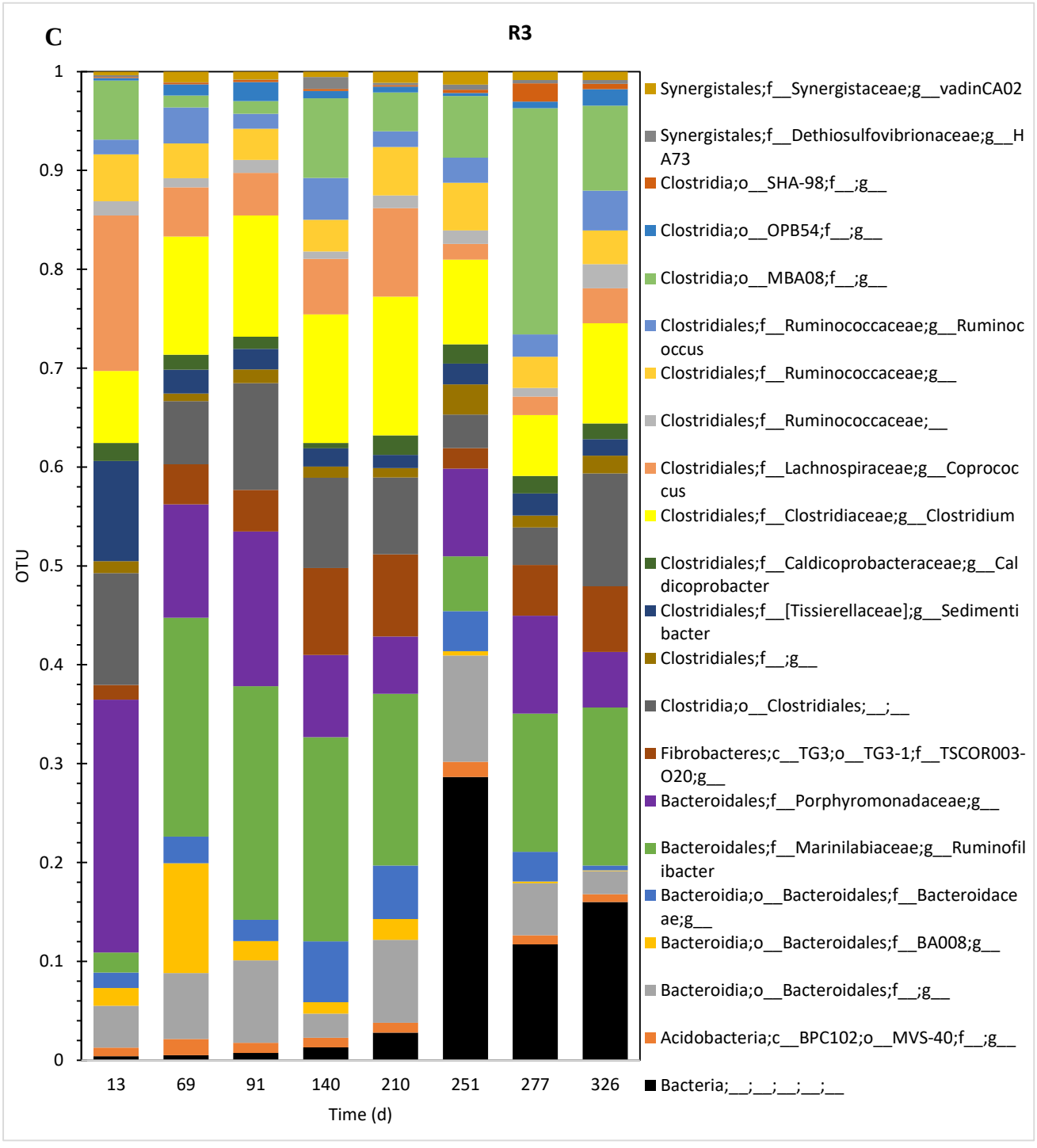
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Synergistales;f__Dethiosulfovibrionaceae;g__HA73
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Clostridia;o__OPB54;f__;g__
Clostridia;o__MBA08;f__;g__
Clostridiales;f__Ruminococcaceae;g__Ruminococcus
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Clostridiales;f__Lachnospiraceae;g__Coprococcus
Clostridiales;f__Clostridiaceae;g__Clostridium
Clostridiales;f__Caldicoprobacteraceae;g__Caldicoprobacter
Clostridiales;f__[Tissierellaceae];g__Sedimentibacter
Clostridiales;f__;g__
Clostridia;o__Clostridiales;__;__
Fibrobacteres;c__TG3;o__TG3-1;f__TSCOR003-O20;g__
Bacteroidales;f__Porphyromonadaceae;g__
Bacteroidales;f__Marinilabiaceae;g__Ruminofilibacter
Bacteroidia;o__Bacteroidales;f__Bacteroidaceae;g__
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Bacteria;__;__;__;__;__

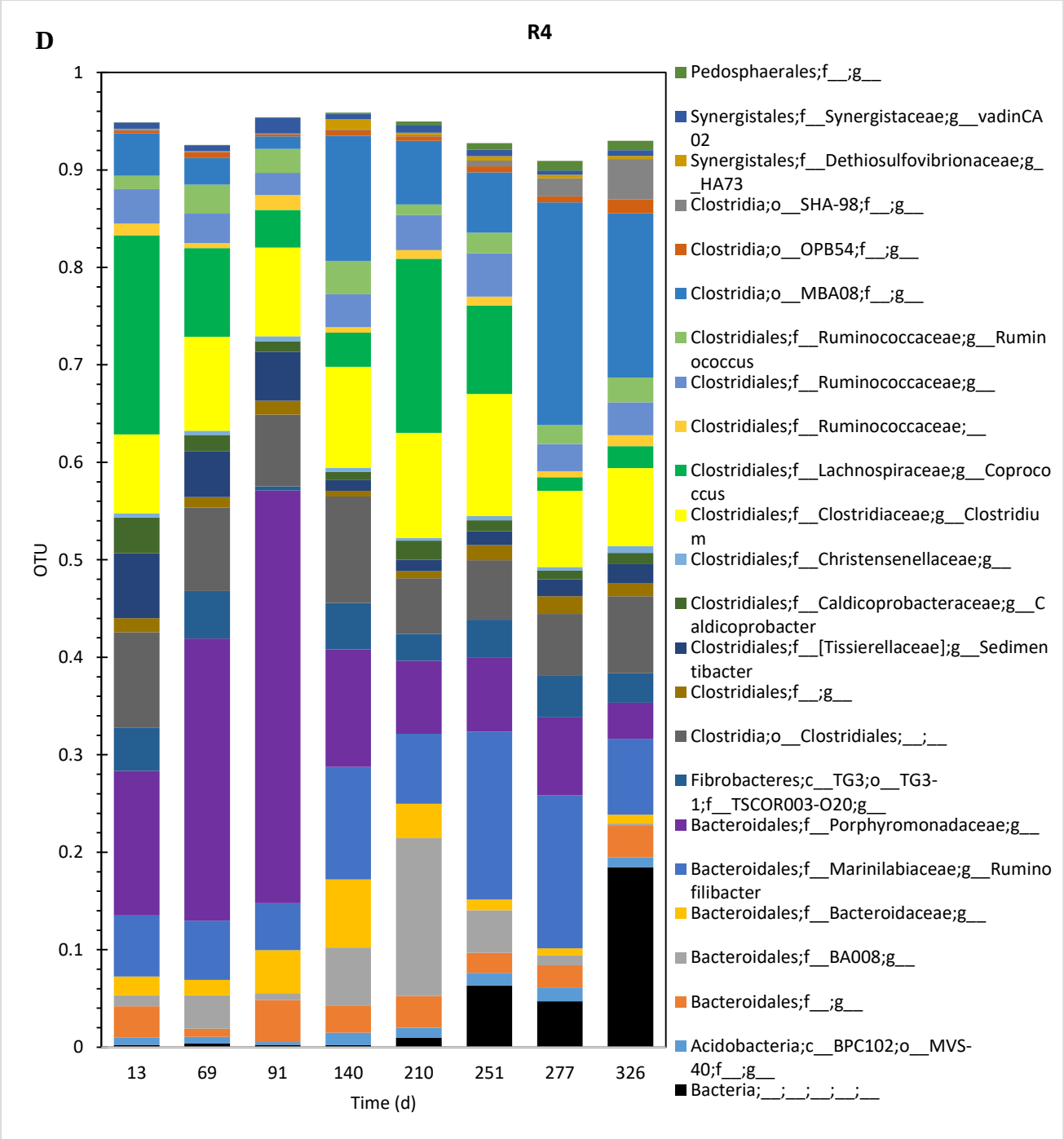


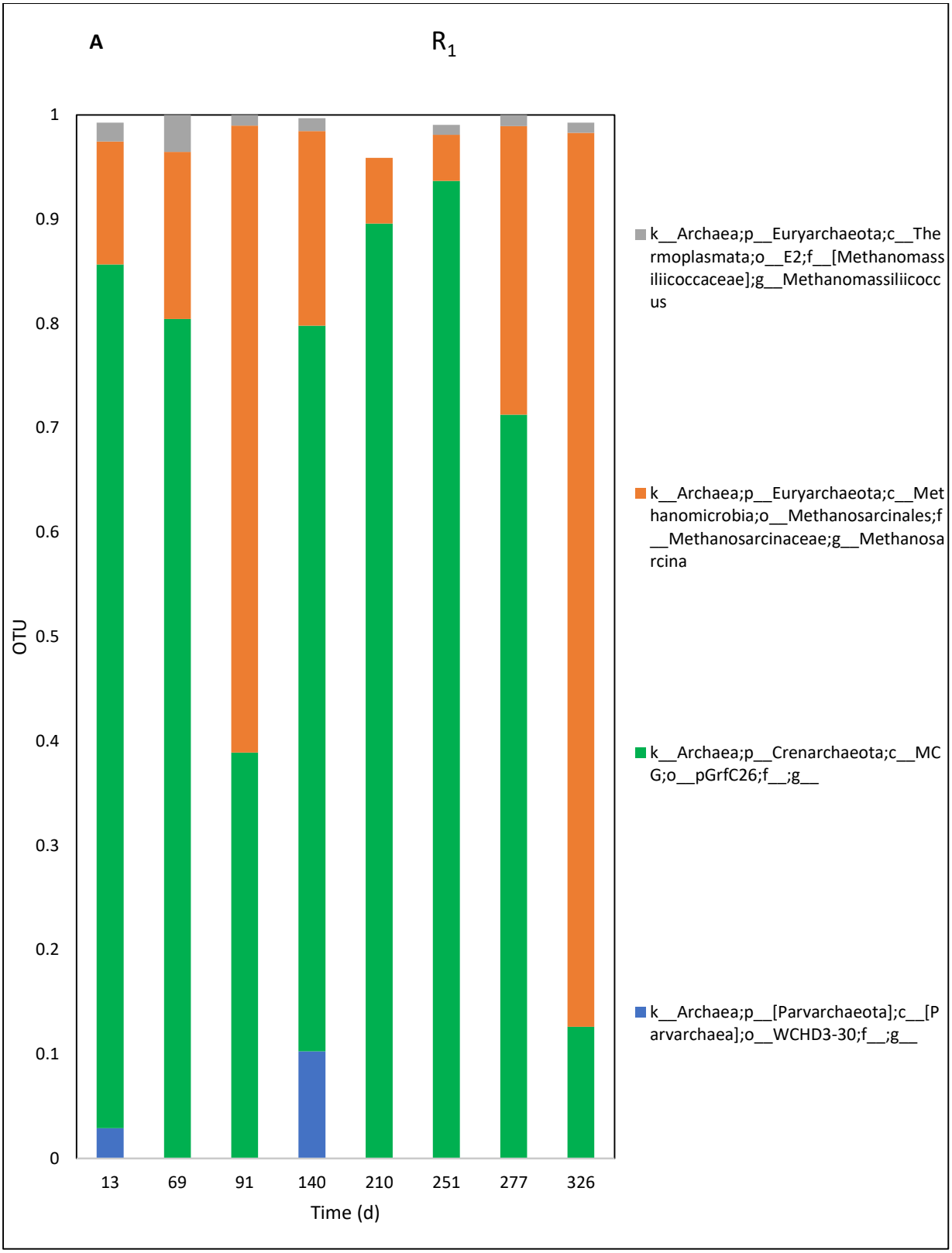
Figure 4-11: The relative abundance of the bacterial populations against the time for bioreactors A: R_1 digesting steam-exploded wheat straw, B: R_2 digesting steam-exploded wheat straw, C: R_3 digesting wheat straw and D: R_4 digesting chopped wheat straw

4.4.7 Archaeal composition

The effect of the pretreatment on the methanogenic populations inside the four bioreactors was also investigated during the present study. Unlike bacteria populations, the diversity of the archaebacteria was limited to up to 4-5 species families per reactor, during the whole experimental period. In the present study, an uncharacterised archaebacterium from the family of *Crenarchaeota* (*Crenarchaeota; c__MCG; o__pGrfC26;f__;g_*) was found to be the most dominant for all anaerobic bioreactors with a maximum presence of up to 97% of the total archaea population. Microorganisms that belong to this family are known to have a predominant role in anoxic environments, including AD systems (Meng et al., 2014). Subsequently, the 2nd most dominant archaeal species belonged to the family of *Methanosarcinaceae* (genus of *Methanosarcina* sp.). Interestingly this family was found in increased numbers in R_1 (SE) for day number 277 (85% w/w) and day number 91 (60 % w/w) when the total VFA increased. Unfortunately, the DNA sequencing report for R_2 revealed the presence of archaea only at the beginning and the end of the experiment and so safe conclusions for this bioreactor cannot be done. Although, the possibility of a total absence of the methanogenic population from the reactor is not feasible due to the continuous biogas production and its measured composition in CH_4 which was found to be higher than 25% (v/v) for the whole experimental period. On the other hand, for R_3 and R_4, the family of *Methanosarcinaceae* was found to numbers up to 16 % (Fig. 11 B, C). All microorganisms that belonged to the genus *Methanosarcina* sp. are also known for their ability to adapt in high-stress AD conditions such as the high presence of ammonia, high concentration of salt and variations of the operational temperature (De Vrieze et al., 2012). Moreover, this type of microorganism is known to multiply fast (1.0–1.2 d) and can withstand changes in the pH of their environment up to 0.8-1.0 pH scale units

according to Conklin et al. (2006). Furthermore, the genus *Methanosarcina* sp. is the only microorganism found in the present study, that belongs to acetoclasts (e.g. microorganisms that reduce CO_2 to CH_4) (Derilus et al., 2019) so the decreased presence in R_3 and R_4 can be attributed to the low accumulation of VFA inside this two reactors (Fig. 5).

Finally, in R_3 and R_4, the presence of the genus *Methanomassiliicoccus* sp. and an unidentified species from the class of Parvarchaea (Parvarchaea]; o__WCHD3-30;f__;g__) was found in numbers between 7% and 20% (w/w) respectively.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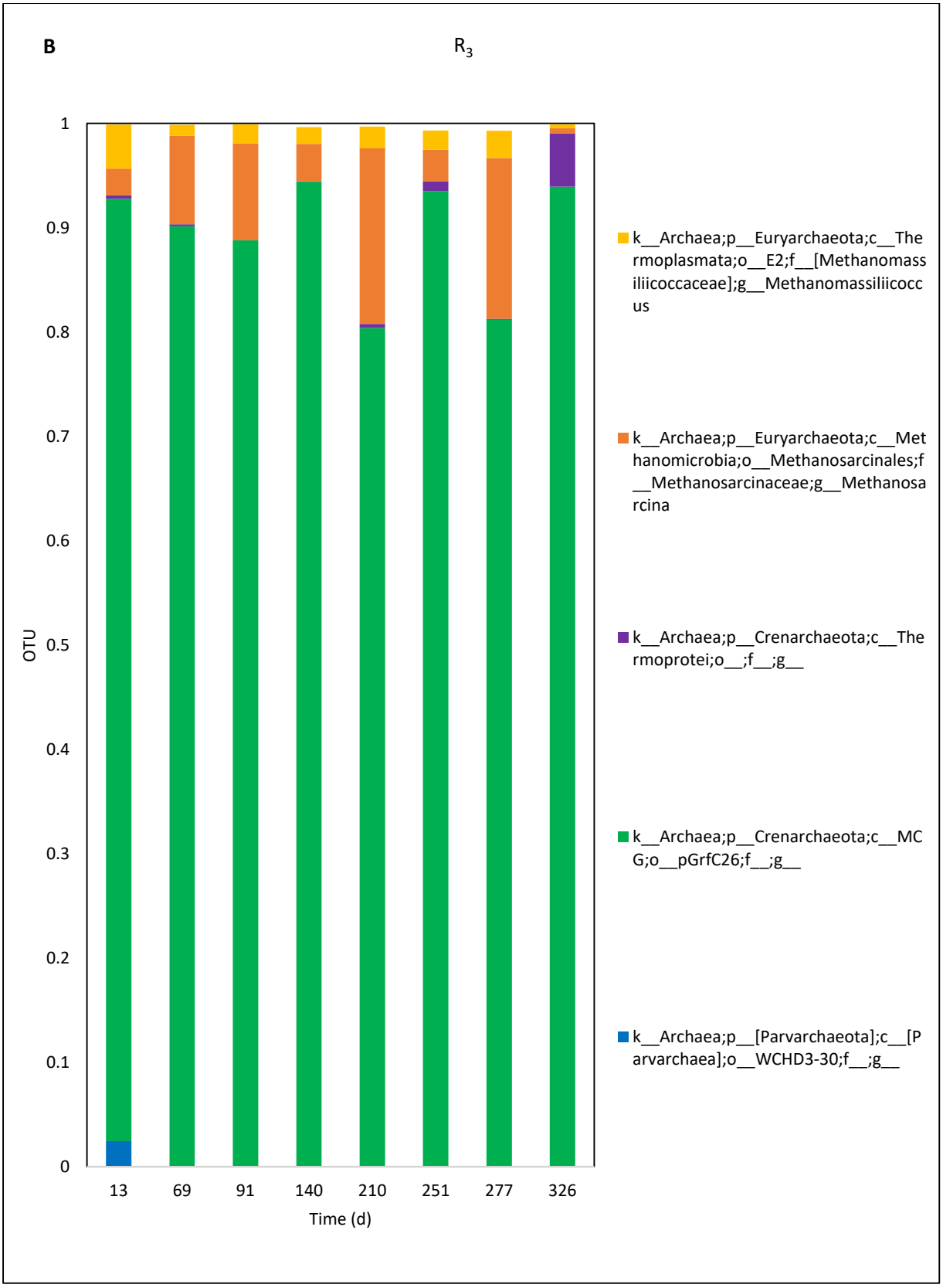
Time (d)
k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae];g__Methanomassiliicoccus
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosarcinaceae;g__Methanosarcina
k__Archaea;p__Crenarchaeota;c__MCG;o__pGrfC26;f__;g__
k__Archaea;p__[Parvarchaeota];c__[Parvarchaea];o__WCHD3-30;f__;g__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Time (d)
k__Archaea;p__Euryarchaeota;c__Thermoplasmata;o__E2;f__[Methanomassiliicoccaceae];g__Methanomassiliicoccus
k__Archaea;p__Euryarchaeota;c__Methanomicrobia;o__Methanosarcinales;f__Methanosarcinaceae;g__Methanosarcina
k__Archaea;p__Crenarchaeota;c__Thermoprotei;o__;f__;g__
k__Archaea;p__Crenarchaeota;c__MCG;o__pGrfC26;f__;g__
k__Archaea;p__[Parvarchaeota];c__[Parvarchaea];o__WCHD3-30;f__;g__

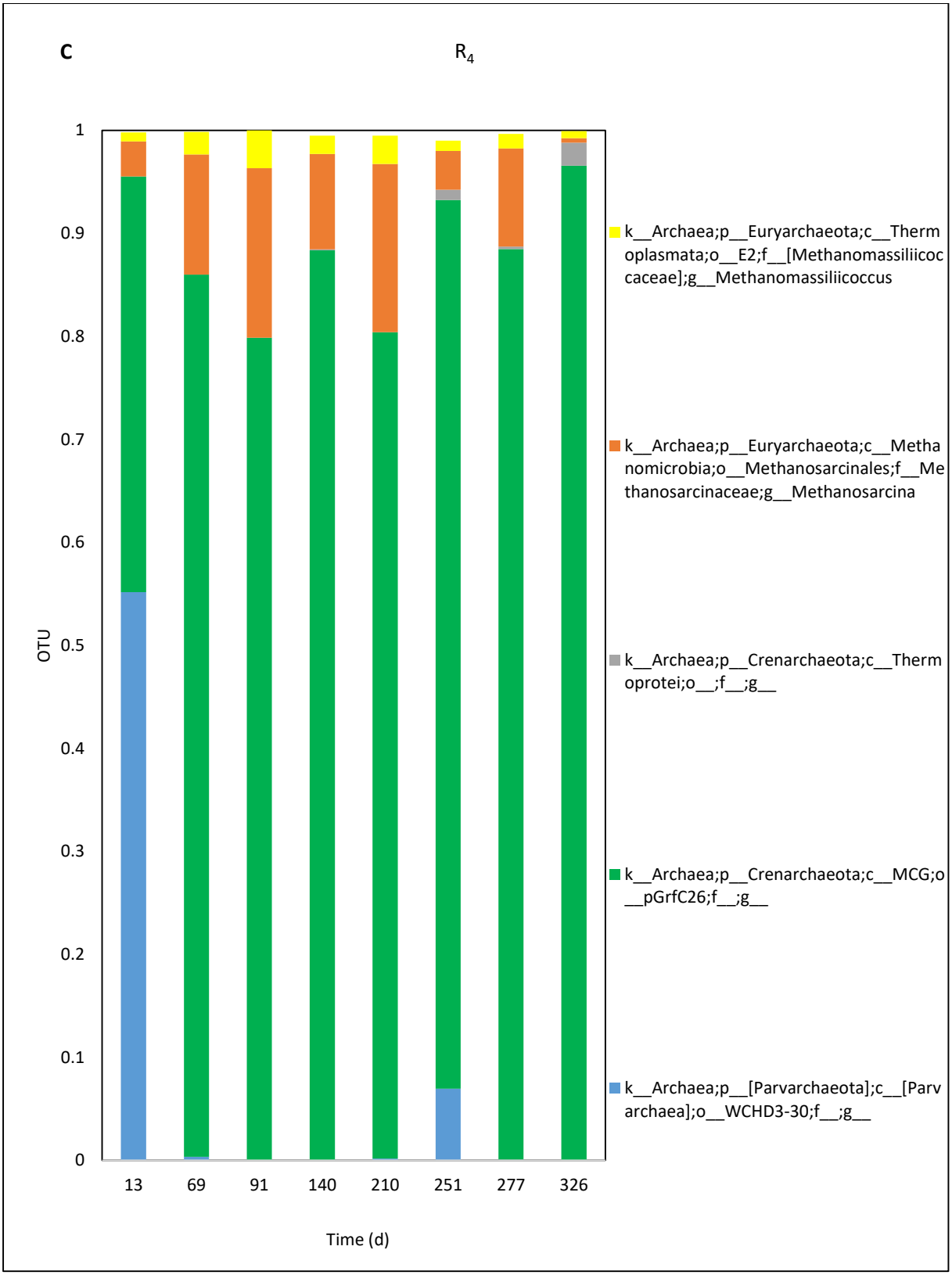

Figure 4-12: The relative abundance of the archaea populations against time for bioreactors A: R_1 digesting steam-exploded wheat straw, B: R_3 digesting wheat straw and D: R_4 digesting chopped wheat straw

4.5 Conclusions

The combination of steam explosion and mechanical pretreatment of WS was evaluated for the first time both in BMP and in CSTR digestion systems. In the BMP experiment, both of the pretreatments can offer a significant improvement on the anaerobic biodegradability of WS, while the combination of the two did not increase the gas yields any further. With regards to the continuous digestion of WS, steam-exploded samples exhibited increased CH_4 yields but at the same time resulted in decreased buffering capacity, rapid production of VFAs and decreased microbial biodiversity. The CSTR system demonstrated an ability to quickly recover from these conditions when the addition of a buffer solution was combined with a partial re-inoculation of the system. Furthermore, according to the microbiological analysis, the steam explosion pretreatment had a higher effect on the microbial populations of AD compared to the mechanical pretreatment. Additional consideration of the financial parameters, for the purchase and the operation of the necessary equipment for the two pretreatments, is also needed for justifying whether one of them is a more sustainable solution towards the increase of WS biomethanation.

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5 Anaerobic digestion of steam-exploded wheat straw and co-digestion strategies for enhancing biogas production

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5.1 Abstract

Biogas represents a renewable energy source that could contribute to the creation of a more sustainable future, independent of fossil fuels. Wheat straw (WS) is a favourable bioenergy substrate because it does not come in direct competition with food or feed crops. However, due to its rigid structure and high C/N ratio, its biodegradability during anaerobic digestion (AD) is usually low. In the present study, the effect of steam explosion pretreatment on WS, combined with the addition of inorganic nitrogen, on biogas production was evaluated. At the same time, co-digestion of WS in various ratios with protein-rich food processing by-products [dried distillers' grains with solubles (DDGS) and rapeseed meal (RM)] was assessed. Steam explosion pretreatment enhanced biogas production from WS, whereas the addition of NH_4Cl was statistically beneficial ($p<0.05$) for the digestion of steam-exploded wheat straw (SE). Furthermore, mono-digestion of the four different substrates seemed to be efficient in both tested organic loading rates (OLR) (6 and 12 g VS/L). Finally, during co-digestion of WS and SE with DDGS and RM, an increase in the cumulative methane production was noted when higher amounts of DDGS and RM were co-digested but the biodegradability of WS and SE was increased when lower amounts of food processing by-products were added as co-substrates.

5.2 Introduction

The transition towards a more sustainable economy, both from an economic and environmental point of view, can be achieved through the replacement of fossil fuel (e.g. coal or petroleum) with renewable alternatives. The reliance on traditional fuels can be decreased along with the increase in the use of biomass-derived biofuels and chemicals. Furthermore, biological products, such as biogas which is the main product of anaerobic digestion (AD) systems, are expected to play important role in the reduction of carbon emissions which is one of the most important causes of global warming. Biogas is a renewable gas, mainly comprising methane (CH_4) and carbon dioxide (CO_2) and can be used for the production of heat and/or electricity. Biogas can also be utilised directly as a fuel for vehicles or following an upgrade to biomethane, it can be injected into the natural gas grid. Energy crops are commonly used today for the production of biofuels, such as biogas and bioethanol (Amon et al., 2007; Barcelos et al., 2016; Zhang et al., 2019). To avoid unwanted competition with crops intended for human consumption, biomass for bioenergy production should derive from non-arable areas.

Alternative materials that can be used as feedstock for AD systems include lignocellulosic residues, such as wheat straw (WS). WS is the most abundant source of biomass in Europe (Kim and Dale, 2004; Talebnia et al., 2010) and the second worldwide after rice straw (Horn et al., 2011). Unfortunately, a high percentage of the annually-produced straw worldwide is not exploited at the maximum possible level. As an example, only China produces between 180 and 280 million tons of rice straw annually and more than half of it is left unused (Zhou et al., 2016). WS is primarily used globally as feed and bedding material for ruminants, however, part of the produced amounts is burned or left unused (Chandra et al., 2012b). Along with its high

availability, its relatively low price is another factor that renders WS an attractive substrate for AD (Fjørtoft et al., 2019). For example, in the UK the market price for a premium quality WS variety would lie within the range of £10-£15/t in 2020 (Farmers Weekly, 2020). However, the anaerobic biodegradability of WS is usually low, due to its rigid structure and its chemical composition. Firstly, WS contains high amounts of non-anaerobically degradable lignin (Fernandes et al., 2009). Lignin is a cross-linked polymer that creates bonds with cellulose and hemicellulose generating a structure that is not easily accessible by the microorganisms of AD. Additionally, the usually low protein content of WS renders it not highly favourable as a sole feedstock for AD systems where no supplementation of additives takes place (L. Liu et al., 2015). An important parameter that limits the anaerobic biodegradation of WS is the ratio of carbon to nitrogen content (C/N) which sometimes is higher than 90 (w/w) for this type of material (L. C. Ferreira et al., 2013; Pohl et al., 2013). Previous studies have already stated that the microorganisms of AD consume carbon faster than nitrogen and the optimal C/N ratio for an AD system is usually within the range of 20 to 30 (w/w) (Estevez et al., 2012).

C/N values lower than the optimal range can cause an increase in total ammonia nitrogen levels and/or high accumulation of volatile fatty acids (VFAs) which can, in turn, inhibit the methanogenesis stage and lead to failure of the whole AD process (Yan et al., 2015). On the other hand, higher C/N values may hold up the microbial growth rate and decrease the biodegradability of the feedstock. As a consequence, when the C/N is not close to the optimal range for AD, biogas production will be limited and the biodegradability of the substrate will be further decreased.

A commonly used strategy for AD efficiency improvement of feedstocks with C/N values outside the optimal range is co-digestion. Different organic materials with high

nitrogen content have been tested as co-substrates along with WS, including food processing by-products (Shi et al., 2018) and various animal wastes, such as chicken manure (Hassan et al., 2016). Furthermore, the addition of inorganic nitrogen (e.g. NH_4Cl) is another way to balance the C/N ratio in AD systems operating with WS as a sole substrate.

Two food processing by-products with high nitrogen content that can be used as a co-substrate in the AD of WS are dried distillers' grains with solubles (DDGS) and rapeseed meal (RM). DDGS is the main by-product of the dry-grind distillation process for the production of alcoholic drinks (whisky) or biofuels (bioethanol) (Chatzifragkou et al., 2016). On the other hand, RM is the main by-product of the rapeseed (*Brassica napus)* oil production process. The main use of these two food processing by-products (FBP) is as livestock feed, while they hold potential as raw materials for the production of high added-value compounds such as biopolymers, platform chemicals and biofuels (Chatzifragkou et al., 2015; Kolesárová et al., 2013). The expected increase in the production of biofuels due to the EU regulations (European Commission, 2018) could potentially result in a subsequent increase in RM and DDGS production. The use of DDGS or RM in AD could provide a market value for their producers through the efficient reuse of these by-products for energy production. RM and DDGS have already been tested as sole substrates in different AD systems (Antonopoulou et al., 2010; Cesaro et al., 2013; Fu and Hu, 2016; Kolesárová et al., 2013; Ziganshin et al., 2011). Up to now, neither DGGS nor RM have been tested as co-substrates with WS in AD systems. For that reason, different co-digestion scenarios with WS, SE, RM and DDGS on varying ratios were evaluated. To this end, untreated and steam explosion pretreated WS were used as main feedstock, in combination with either inorganic nitrogen source or RM and DDGS, to evaluate their effect on biogas production in AD.

5.3 Materials and methods

5.3.1 Substrates and inoculum

Three different and independent batch experiments were conducted with four different materials as feedstock. The four different feedstocks were the untreated WS, steam-exploded wheat straw (SE), dried distillers' grains with solubles (DDGS) and rapeseed meal (RM). DDGS was supplied from a UK bioethanol plant (Vivergo, Yorkshire, UK), was ground into a fine powder using a coffee grinder (DeLonghi, Australia), sieved through the sieve mesh No. 20 (particle size smaller than 0.85 mm) and stored at room temperature (20 °C) until further use. RM was kindly provided by Stainswick Farm (Oxfordshire, UK) and was generated via cold pressing oil extraction process. RM samples were ground using a dry-grinder and sieved to obtain uniform sized particles (<850 μm). The remaining oil in the meal was removed using a supercritical CO_2 extraction rig (SciMed, UK) at 60 °C and 300 bar pressure for 1 h, with ethanol (10%, v/v) as co-solvent. The residual defatted meal was kept at 4 °C prior to use. WS was collected from fields in the wider area of Northfolk, UK. Part of this residue was steam-exploded with the use of an economiser (Economizer SE, Biogas Systems, Austria). The Economizer was fed with raw WS and operated at high temperature (155°C) and pressure (5 bar) for 3 min with subsequent release of generated pressure by a valve causing rapid depressurization of the substrate. Pretreated WS was stored in plastic bags and was kept frozen at -20°C until further use.

As inoculum source, the effluent of four lab-scale mesophilic (42±1°C) continuous stirring tank reactors (CSTR) with a working volume of 4 L, operating in a steady-state at an OLR of 5 g VS/L per day, was used. Two of the CSTR reactors were digesting untreated WS while the other pair was fed with steam-exploded WS. The reason for

mixing two slightly different inocula was to minimize as much as possible the chances of having an inoculum acclimatized to one of the two substrates (raw WS vs SE). Equal amounts of the two effluents (2 litres of each inoculum) were manually mixed and degassed at mesophilic conditions (42±1°C) for one week before the beginning of the AD experiments to minimize the endogenous microbial activity.

5.3.2 Analytical techniques

Total solids (TS) were determined in triplicate for WS, SE, DDGS, RM and the inoculum, according to the protocol described in APHA (2005). Briefly, samples were dried at 105°C (Gallenkamp, UK) overnight and subsequently ignited at 550°C using a muffle furnace (Carbolite, Sheffield England) for 5 h. The volatile solids (VS) content was calculated as the difference between TS content and the produced ash (after the 550°C drying), divided by the weight of the wet sample, in accordance with standard methods for the examination of water and wastewater (APHA., 2005). The TS and VS content was calculated based on the following equations Eq.5-1 and Eq.5- 2, respectively:

Equation 5-1: Total Solids $= \frac{(crucible\ net\ weight + dry\ mass) - (crucible\ net\ weight)}{(crucible\ net\ weight + wet\ mass) - (crucible\ net\ weight)} \times 100$

Equation 5-2: Volatile Solids $= \frac{(crucible\ net\ weight + dry\ mass) - (crucible\ net\ weight + ash)}{(crucible\ net\ weight + wet\ mass) - (crucible\ net\ weight)} \times 100$

The crude protein content for SE, WS, DDGS and RM was also measured using the Kjeldahl method as described before (APHA., 2005). Lignin, hemicellulose and cellulose contents for the four substrates were measured using the NREL protocol proposed by Sluiter et al. (2012) as previously described in Chapter 3 of this thesis.

Finally, the presence of carbon in WS, SE, DDGS and RM was calculated based on the composition of the four feedstocks in carbohydrates and proteins with the assumption of all carbohydrates as glucose ($C_6H_{12}O_6$) and all proteins as gluten ($C_{29}H_{45}N_5O_8$).

5.4 Biochemical Methane Potential tests

The biochemical methane potential tests (BMPs) is a commonly used method to determine the anaerobic biodegradability of different organic materials and their potential to produce biogas (Strömberg et al., 2014). In this study, 150 mL serum glass vials with a working volume of 70 mL were utilised across three batches of experiments with an incubation period of 30 d for each experiment at mesophilic conditions (42±1°C). During the first experiment, inorganic nitrogen (NH_4Cl) was added in the system to adjust the C/N ratio from 88 and 64, for WS and SE respectively, to 30 (w/w) for both feedstocks, alongside bottles with no added nitrogen (controls), at an organic loading rate (OLR) of 6 g VS/L. The second experiment comprised two different OLR's (6 and 12 g VS/L) across separate WS, SE, DDGS and RM substrates. Finally, the two different straw samples (e.g. untreated and steam exploded straw) were mixed on ratios of 50:50, 70:30 and 90:10 (w/w) with DDGS and RM, respectively. Based on the results of the second experiment, the OLR for the third batch trial was also set at 12 g VS/L. For all three experiments, methane production was measured daily by applying the liquid displacement method, while NaOH (aq) was used for scrubbing the CO_2 from the produced biogas. All BMP variations were performed in at least a triplicate alongside blank and control trials. The blanks were used for measuring the endogenous methane production, while the obtained values were subtracted from those acquired from the vials with the substrate. In addition to the blank vials, during all experimental setups, control samples with microcrystalline cellulose (Avicel) along with the

inoculum were used as a positive control as suggested before (Flores et al., 2015). All BMP vials were manually shaken every 12 h. Finally, all methane measurements were reported and presented after correction to normal conditions based on the Eq. 5-3:

Equation 5-3: $CH4n = \frac{CH4e \times K}{(K+T)}$

where, CH_4 n: total methane production at normal conditions, CH_4 e: experimentally measured methane values, T: room temperature at the point of the measurement and K equals 273.15 and is used to express the temperature in Kelvin rather than Celsius degrees.

5.5 Statistical analyses

The statistical analyses were conducted on Excel software (Microsoft Office 365 ProPlus, version 1908) with a paired student's t-test and the statistical significance assigned to $p<0.05$.

5.6 Results and discussion

5.6.1 Physicochemical characterisation of the feedstock

Total solids analysis showed that the steam explosion pretreatment affected the solids concentration in WS. More specifically, the TS content decreased from 36 % in WS to 21.5% (w/w) in SE (Table 5-1). Similarly, SE had a VS content of 19.37 % (w/w) while in WS, VS accounted for 33.38% (w/w). Before the beginning of the pretreatment, water was added to the pretreatment vessel so the feedstock can be pumped out after the steam explosion process. It is likely that the increased temperature during SE pretreatment (155 °C), allowed the pores of the substrate to open and absorb moisture. Similar results were reported in previous studies examining the effect of steam explosion pretreatment on the AD of WS (Theuretzbacher et al., 2015b). DDGS and

RM were found to have similar VS content of 86.1% (w/w) and 86.36% (w/w) respectively, whereas the inoculum contained 2.53 % VS (w/w). Also, the steam explosion pretreatment seemed to increase the percentage of cellulose content in the WS, from 37.41 to 47.9 % (w/w) of the total dry biomass. These results might not be in agreement with studies conducted in the past where is pointed out that cellulose content in lignocellulosic residues is not following a specific trend after steam explosion pretreatment (Menardo et al., 2013; Theuretzbacher et al., 2015b). However, differences in the configuration of cellulose (e.g. crystalline and amorphous structures) and the crystallinity index (the relative amount of crystalline structures in cellulose) within different lignocellulosic residues might affect the response of this material to steam explosion pretreatment according to Lizasoain et al. (2016).

Unlike cellulose, the percentage of hemicellulose in WS samples did not seem to be highly affected by the application of the pretreatment. However, this result can be attributed to the fact that the applied steam explosion temperature (155°C) was relatively low for hemicellulose hydrolysis to occur (between 150 and 230°C) according to Garrote et al. (1999). Even if a percentage of hemicellulose was solubilized during SE, no washing step was followed resulting in the hydrolysed portion of hemicellulosic sugars remaining in the solids effluent. The reason for avoiding the washing of the material was to simulate a situation where an AD system is fed directly with the effluent of the steam explosion equipment. In addition to that, total solubilisation of hemicellulosic substances would not be highly preferable, since oligosaccharides derived from hemicellulose can also be used by the anaerobic microorganisms as a substrate for biogas production. The analytical measurements also confirmed the higher protein content of DDGS and RM, compared to WS and SE, representing 28% and 25% (w/w), respectively for the two FBP. At the same time, crude

protein content was lower than 1% (w/w) for both straw samples. On the other hand, both DDGS and RM found to contain a significantly lower concentration of lignin than WS samples (Table 5-1), which was expected since both DDGS and RM derive from the grain of their respective plant (wheat or corn and rapeseed), while straw is the stem part of wheat where lignin is a structural element supporting the plant. Summarising, steam explosion pretreatment as conducted for the needs of the present study did not have a significant effect on the composition of WS with only an exception of an increase in the cellulose and protein content.

Table 5-1: Physicochemical characteristics of the feedstock and the inoculum that was used in the present study. * Values presented on a % (w/w) on dry matter basis

%, w/w (DM)	**Wheat straw (WS)**	**Steam exploded straw (SE)**	**DDGS**	**RM**	**Inoculum**
TS	36.06±0.52	21.04±0.57	91.46±0.29	92.42±0.65	3.37±0.13
VS	33.38±0.63	19.37±0.53	86.1±0.34	86.39±0.68	2.53±0.11
Crude protein	0.29±0.01	0.47±0.02	28.3±0.5	25.28±0.15	n.d.
Cellulose (glucose)	37.41±0.77	47.92±0.50	11.1±0.4	20.17±2.32	n.d.
Hemicellulose	27.56±0.75	28.23±0.12	20.3±1.7	14.03±2.03	n.d.
Acid insoluble lignin	28.09±2.6	25.28±1.68	n.d.	16.08±0.15	n.d.
Acid soluble lignin	2.38±0.02	2.05±0.11	2.9±0.1	1.9±0.1	n.d.

*All data were produced in duplicate and the standard deviations are presented.

5.6.2 BMP – Inorganic nitrogen addition

In the first BMP series, the effect of steam explosion pretreatment together with the addition of inorganic nitrogen was evaluated. After 30 d of BMP digestion, untreated WS presented an average cumulative methane production of 280 mL CH_4/g VS. Similar values in methane production (304.29 mL/g VS) for untreated WS in BMPs have been reported elsewhere (Pohl et al., 2013). The slightly higher methane yields in the study of Pohl et al. (2013) could be attributed to higher culture temperatures (55 °C) compared to the mesophilic system (42 °C) used in the present study. In the same study, the digestion of WS was also evaluated in a continuous digestion system and it was found

that the addition of nitrogen on the long term AD of WS was also necessary for the stability of the whole process, as considerable amounts were lost in the digestate (in the case of continuous operation). In the present study, the methane production from SE samples, without nitrogen addition, reached values of up to 332 mL CH_4/gVS after the end of the 30 days digestion period, but this yield was not significantly higher ($p<0.05$) than that from untreated WS without the addition of nitrogen (280 mL CH_4/gVS) (Fig.1). In contrast, the biomethane yields offered by SE BMPs after the NH_4Cl supplementation (SE+N) reached values of 387 mL CH_4/gVS, which were significantly higher than those from WS with the supplementation of nitrogen (WS+N) (302 mL CH_4/gVS) as well as those from SE without N_2 addition (332 mL CH_4/gVS) (Fig.1). It is possible that higher gas yields were the result of an enhanced microbial activity due to the pretreated straw offering increased accessibility to available carbon and with the concomitant increased availability of nitrogen in the culture.

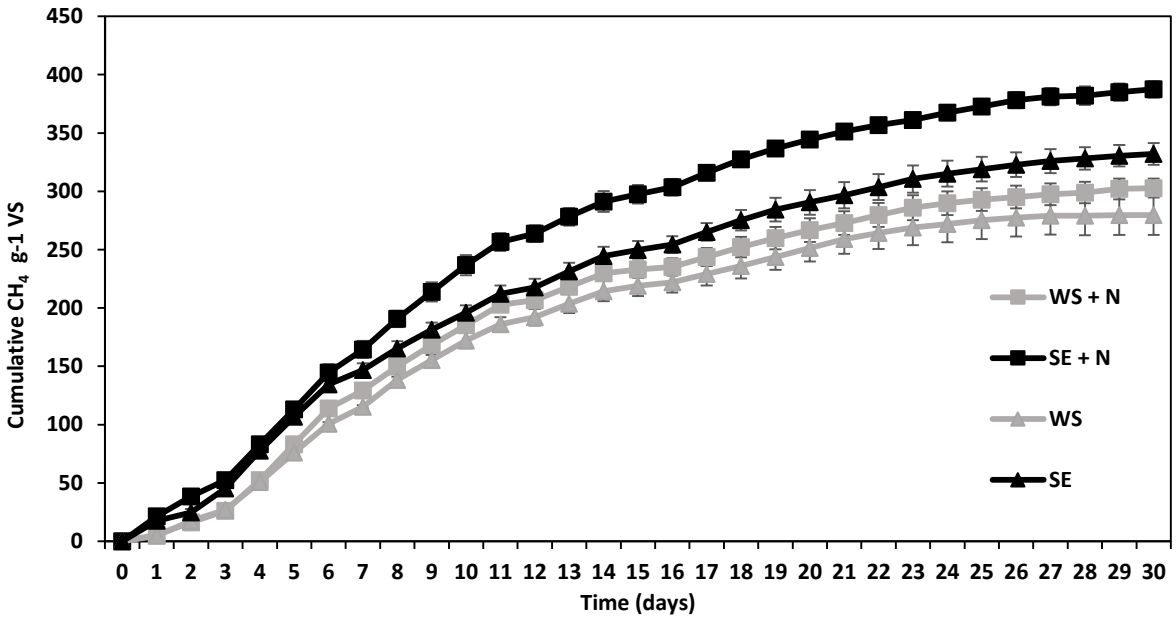


Figure 5-1: Cumulative methane production examining the effect of NH4Cl addition to untreated and steam-exploded wheat straw samples. WS+N: wheat straw with the addition of NH4Cl, SE+N: steam-exploded wheat straw with the addition of NH4Cl, WS: wheat straw, SE: steam-exploded wheat straw

5.6.3 BMP- Comparison of WS, RM and DDGS as AD feedstock

On the second BMP experiment of the present study, four different lignocellulosic residues were examined for their anaerobic biodegradability at two different organic load rates (OLR), namely 6 and 12 g VS/L. The C/N ratio was calculated to be 88 and 64 for WS and SE respectively, while for both DDGS and RM it was ~ 9 (w/w). Despite the low C/N ratio, the digestion of all four different feedstock types was efficient for both tested OLRs, while the higher feeding rate seemed to be more favourable in terms of gas yields offered for all examined feedstocks (Fig 5-2a and 5-2b). DDGS and RM had a higher biomethane potential compared to WS and SE, while no inhibition seemed to occur as a result of their high nitrogen content. However, it is expected that in long term digestion of any of these two feedstocks without a prior balancing of the C/N, the

AD system would not be biologically sustainable due to ammonium accumulation (Kolesárová et al., 2013). In the present study, the highest produced gas yields for the DDGS and the RM were 445 and 405 mL CH_4/g VS respectively both at an organic loading rate of 12 g VS/L (Fig. 5-2 A, B). These results are in agreement with previous scientific publications where both DDGS and RM were evaluated as substrates for BMP systems (Antonopoulou et al., 2010; Gyenge et al., 2013). Specifically, with regards to the digestion of RM, the methane production was close to the maximum theoretical for this feedstock (450 mL CH_4/g VS) according to Kolesárová et al. (2013). The significantly lower concentration of lignin and higher presence of hemicellulosic carbohydrates that are present in these two materials (Table 5.-1), can partially explain the higher methane production from DDGS and RM compared to WS and SE. Except for lignin, cellulose and hemicellulose are the macromolecules found in the highest concentration both in WS and SE. On the other hand, RM and DDGS are also consisting of other, more assimilable carbohydrates, such as pectins present in RM (Harith et al., 2019) as well as beta-glucans in DDGS (Chatzifragkou et al., 2015). It is possible that due to the differences in the hemicellulosic carbohydrates of the feedstock, the microorganisms of the system had increased affinity towards pectins or beta-glucans, as opposed to arabinoxylans in straw, leading to enhanced biomethanation.

In addition to the above, the high protein content in DDGS and RM (Table 5-1), in comparison to WS and SE where protein content was below 1% (w/w), can also explain the improvement of methane production from these two materials. More precisely, the C/N ratio for WS and SE was found to be 88 and 64 (w/w) respectively, while for DDGS and RM the same ratio was calculated to be ~ 9 (w/w). The presence of nitrogen in AD systems, not only balances the C/N ratio but also according to Angelidaki and Sanders (2004), the methane potential at standard temperature and pressure conditions

(STP) for proteins is higher (0.496 L CH_4/g VS) compared to carbohydrates (0.415 L CH_4/g VS). As a consequence, the increased availability of nitrogen inside an AD system is expected to increase the biomethane yields of the system, but only in cases where ammonium levels in the system do not exceed the AD inhibition levels (Kolesárová et al., 2013).

With respect to methane production, the digestion of DDGS, RM, WS and SE at an OLR of 12 gVS/L offered higher yields up to 14%, compared to OLR 6 gVS/L. AD microorganisms usually utilise the available carbon and nitrogen sources in the feedstock to produce biogas and produce energy for maintenance. At the same time, part of the available carbon and nitrogen is channelled towards microbial proliferation. It is possible that the increased availability of the feedstock in the cases of the OLR 12 g/L resulted also in an increase in the microbial population and as a result, the produced gas yields were also increased.

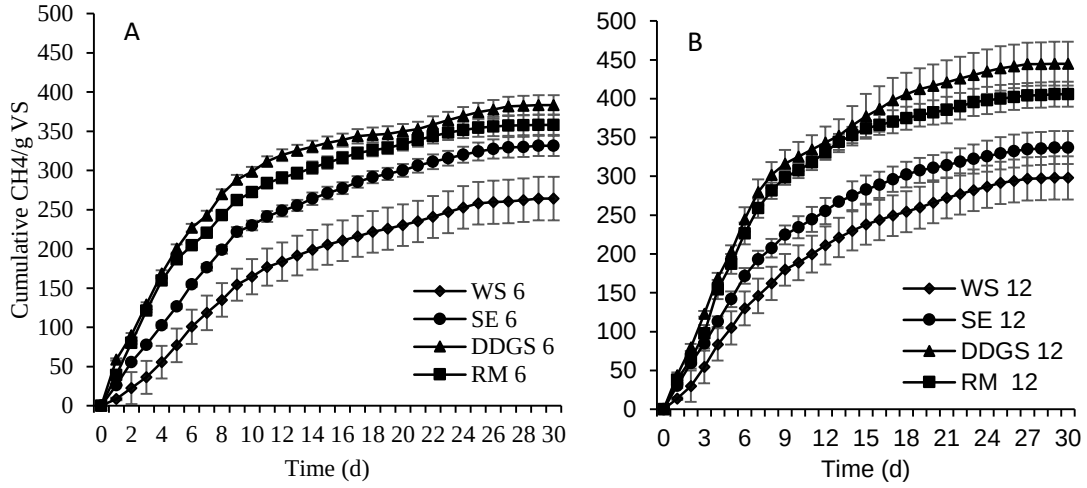


Figure 5-2: Cumulative methane production examining the anaerobic digestion of wheat straw (WS), steam-exploded wheat straw (SE), DDGS and RM at (A) OLR of 6 g VS/L and (B) 12 g VS/l.

5.6.4 BMP- Co-digestion scenarios

Finally, the effect of different co-digestion scenarios between the two straw samples (WS and SE) and DDGS or RM was evaluated in the last BMP experiment of this study. In more detail, WS and SE samples were co-digested with DDGS and RM in three different ratios based on the VS content for the four AD feedstock (50:50, 70:0 and 90:0 w/w). The C/N ratio was calculated for all co-digestion scenarios, based on the composition of each feedstock, and is presented in table 5-2 along with the ratio of each feedstock used in each co-digestion trial.

Table 5-2: Co-digestion scenarios and C/N ratio calculated for all BMP trials

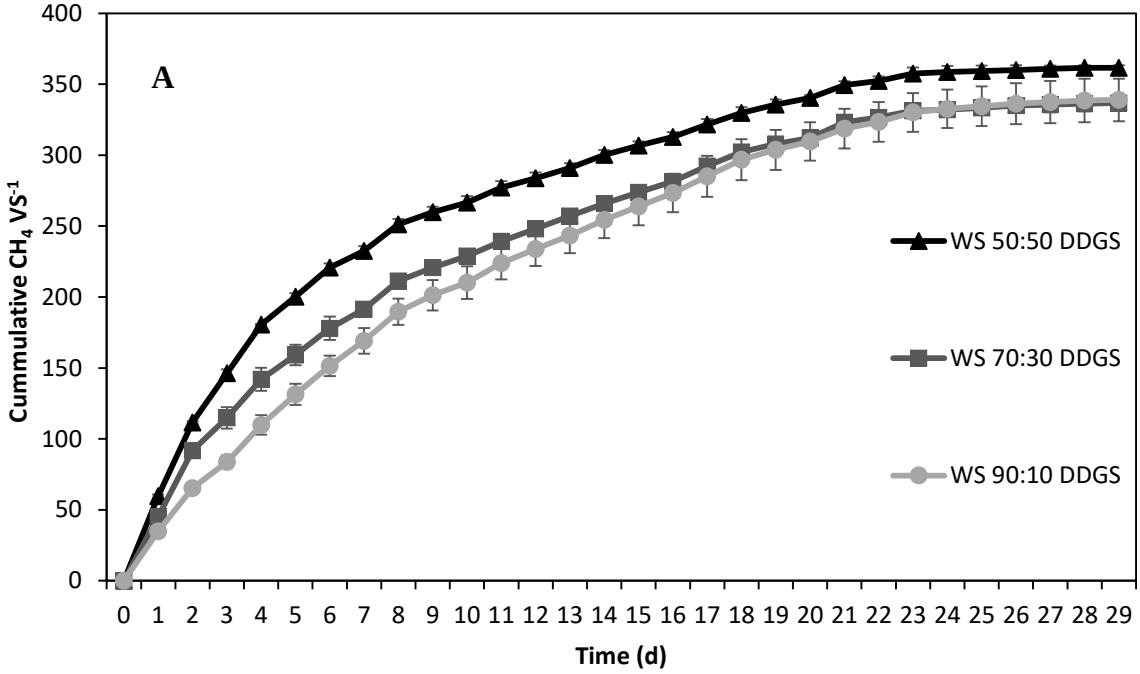
	WS (%)	SE (%)	DDGS (%)	RM (%)	C/N (w/w)
WS-DDGS	50	-	50	-	21
WS-DDGS	70	-	30	-	26
WS-DDGS	90	-	10	-	30
WS-RM	50	-	-	50	19
WS-RM	70	-	-	30	24
WS-RM	90	-	-	10	30
SE-DDGS	-	50	50	-	15
SE-DDGS	-	70	30	-	24
SE-DDGS	-	90	10	-	35
SE-RM	-	50	-	50	15
SE-RM	-	70	-	30	25
SE-RM	-	90	-	10	35

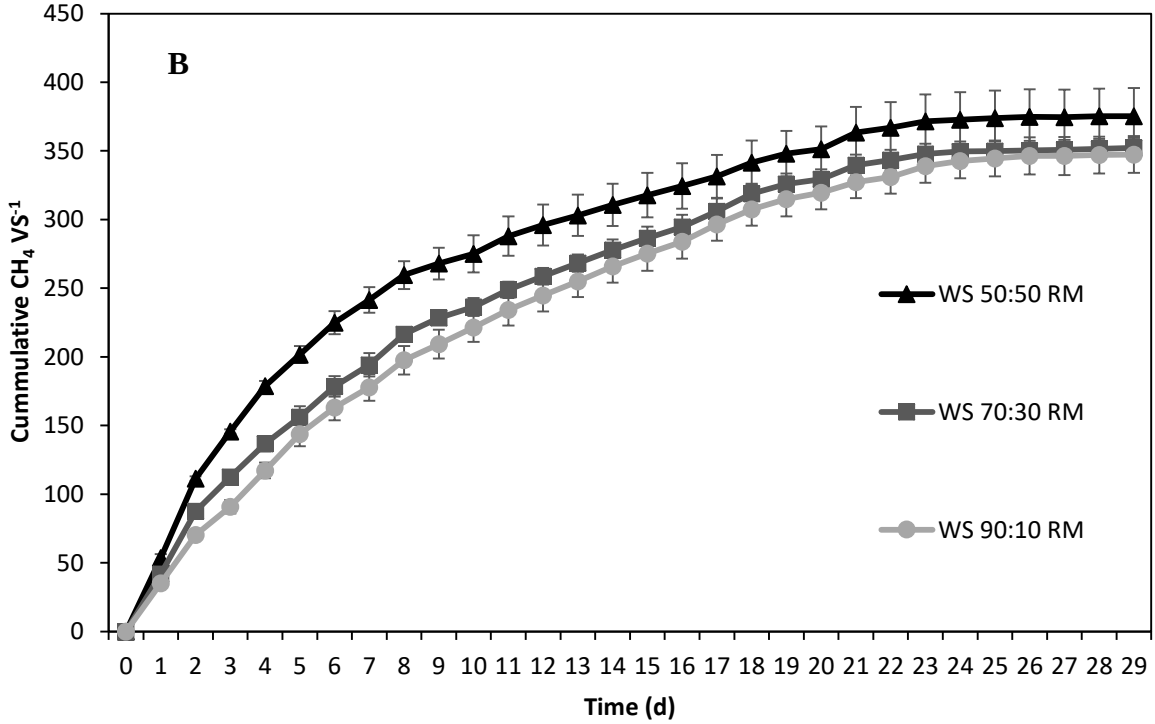
As can be seen in Fig. 5-3 (A-D), the 50:50 co-digestion scenario offered higher, but not statistically significant, methane yields compared to the rest of the trials (70:30 and 90:10 w/w) for both WS and SE co-digested either with DDGS or RM.

With regards to the effect of the type of co-substrate on the AD of WS, for all examined co-digestion ratios it was found a non-significant difference between the production from WS-DDGS (338-361 mL CH_4/g VS) and WS-RM (347-375 mL CH_4/g VS; Fig. 5-3 A-B). Similarly for SE, a co-digestion with DDGS at 50:50 (w/w) resulted in 377 mL CH_4/g VS while for the same ratio the co-digestion of SE with RM offered

comparable biomethane yields of 373 mL CH_4/g VS. However, in SE trials with less DDGS or RM (ratio 90:10), the produced methane yields were significantly higher in DDGS co-digestions compared to RM co-digestion (Fig 5-3 C-D) while very similar C/N ratio (35) was applied in both cases. According to the results from the mono-digestion BMP experiment, DDGS offered slightly higher methane yields as a sole substrate compared to the RM. This higher biogas potential of DDGs compared to RM can potentially explain the increased yields offered by SE co-digested with DDGS compared to SE-RM at the 90:10 ratio.

It is also worth mentioning that, nevertheless the improvement that steam explosion can offer to the digestion of lignocellulosic biomass when WS and SE are co-digested with a food processing by-product, the biomethane potential for the two feedstock is very similar (Fig. 3 A-D). As an example, the highest methane production for WS reported when this feedstock co-digested with RM at a 50:50 ratio (375 ml CH_4/g VS). Similarly, when SE co-digested with DDGS at 50:50 the biomethane outcome reached values close to 377 ml CH_4/g VS. DDGS and RM perform similarly when used as co-substrates in AD with DDGS showing slightly higher biomethane potentials compared to RM. As a consequence, the choice between the two FBPs as a co-substrate in AD can be based on the availability and the price of DDGS and RM without this affecting the operation of the AD system.


A
Cummulative CH_4 VS^{-1}
Time (d)
WS 50:50 DDGS
WS 70:30 DDGS
WS 90:10 DDGS


B
Cummulative CH_4 VS^{-1}
Time (d)
WS 50:50 RM
WS 70:30 RM
WS 90:10 RM

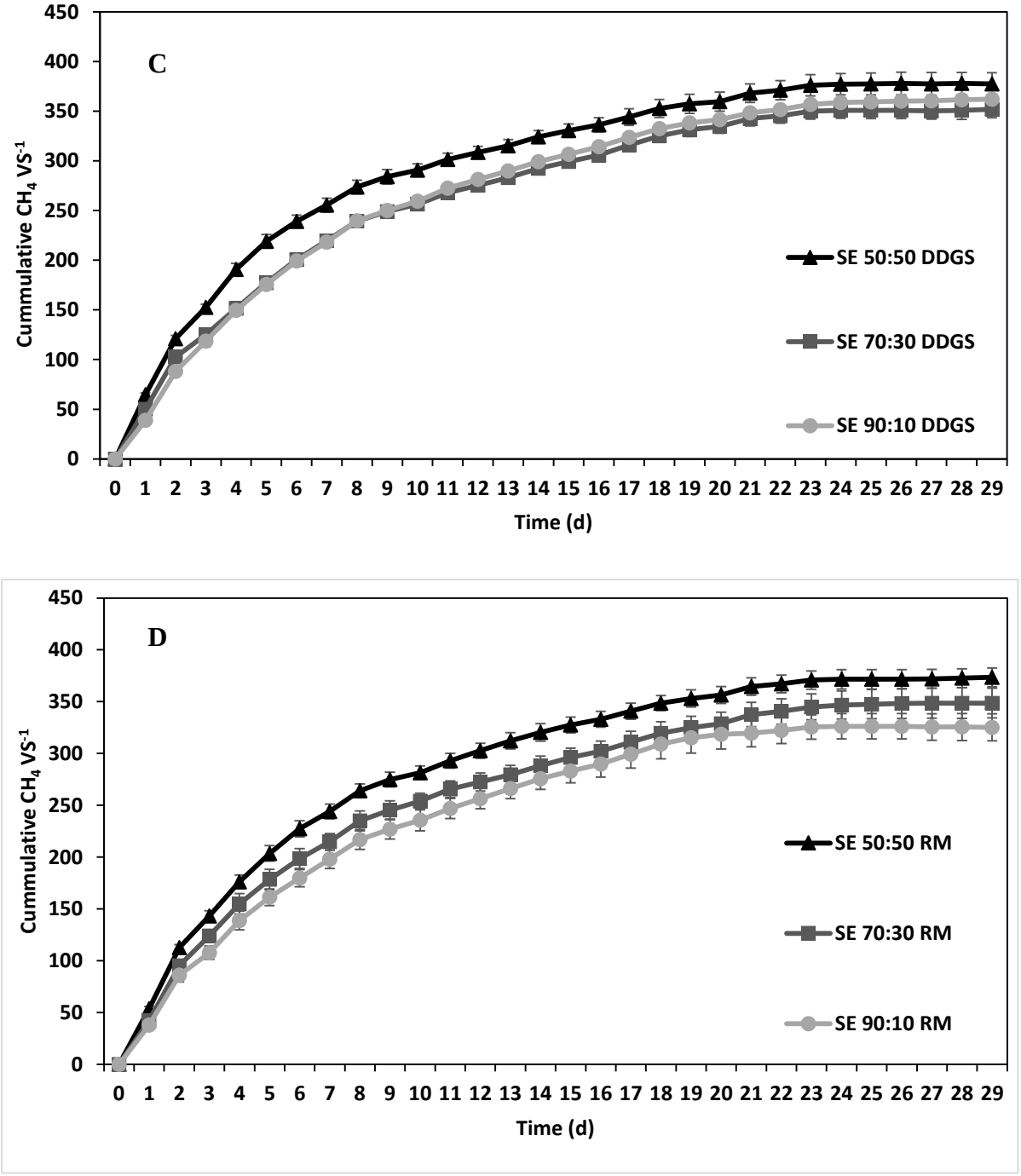


Figure 5-3:Cumulative methane production examining the co-digestion of: (A) Untreated wheat straw with DDGS; (B)Untreated wheat straw with RM; (C) Steam-exploded wheat straw with DDGS and (D) Steam-exploded straw with RM.

Furthermore, to evaluate the effect of co-digestion on the gas production solely from WS and SE straw, methane concentrations recorded in mono-digestion trials of DDGS and RM were subtracted from the cumulative final production of the six co-digestion

scenarios (Fig. 4). Despite the notable increase in gas production in DDGS and RM co-digestion at 50:50, the produced yields from SE seemed to be significantly increased when lower amounts of either DDGS or RM were added to the system (Fig. 4). This observation could be explained by the differences in the C/N between the different BMP trials (Table 5-2). The C/N ratio was calculated within the range of 12 to 15 in all cases were a 50:50 digestion was applied (Table 5-2). At the same time, C/N reached values higher than 35 (w/w) when SE was digested either with RM or DDGS at a 90:10 ratio (Table 5-2). Generally, the produced gas yields for SE were significantly increased as the concentrations of the two food processing by-products decreased. The same increasing trend was observed for WS, however, the difference in the produced yields from the different co-digestion trials was not always significant. As an example, the methane production from WS, after subtracting the methane production from RM (70:30 ratio), was 229 mL CH_4/g VS while insignificantly increased to levels close to 230 mL CH_4/g VS when a 90:10 digestion with RM was attempted. These results can also be attributed to the C/N ratio while for the different co-digestion scenarios between WS and DDGS/RM this ratio was calculated to lie withing the theoretical optimal range for AD (20 and 30) (Table 5-2). Based on the results from the first BMP experiment, the addition of a nitrogen source affected to a higher extend the digestion of SE compared to WS nevertheless the already lower values of C/N that this feedstock offers to the system [e.g. 64 (w/w)]. As reported in previous chapters of the present PhD, the steam explosion pretreatment can partially disrupt the structure of straw and as a consequence, the carbohydrates present in the lignocellulosic biomass become more accessible to the microorganisms of AD. It is possible that this increased availability of degradable components of straw along with the increased availability of nitrogen

resulted in an increase in the microbial population of the system and as a consequence, the biotransformation of the feedstock to biogas was also enhanced.

Another possible explanation for the results presented in Fig. 4 is the selective consumption of the feedstock by AD microorganisms. It is already proven that when different feedstock, or feedstock components, co-exist inside a bioreactor, the microorganisms will firstly consume the easier degradable material (Yang et al., 2015). As shown earlier, the digestion of DDGS or RM offers higher biomethane yields, compared to WS and SE, without any signs of inhibition or lag phase occurring. It is likely that DDGS and RM were more preferable substrates for AD microorganisms and their depletion coincided with micronutrient depletion. As such WS and SE digestion was limited in these cases. On the other hand, when significantly lower amounts of DDGS and RM were present in the AD system, they offered a balanced C/N ratio, enhanced the metabolic activity and were quickly depleted, allowing for a further breakdown of WS and SE.

According to these results, small amounts of either DDGS or RM can be used as an AD supplement to stimulate gas production and improve straw biodegradability. Considering also the increased price of the two food processing by-products compared to WS and SE, a reduced need for DDGS and RM addition would also contribute to decreasing the operational cost for a biogas plant fed with the above feedstocks.

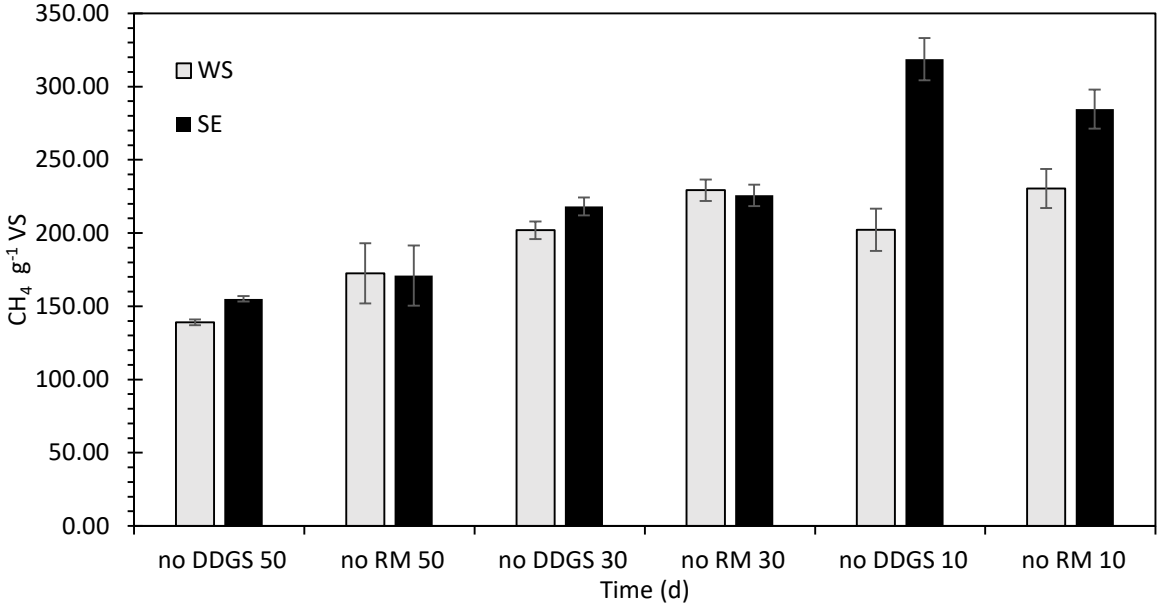


Figure 5-4: Methane production after 30 days of co-digestion of untreated (WS) and the steam-exploded (SE) wheat straw after subtracting the expected methane production of the RM and DDGS respectively

5.7 Conclusions

The effect of steam explosion pretreatment of WS on AD was evaluated, together with an investigation of the provision of nitrogen to balance the high C/N ratio of WS. The steam explosion pretreatment offered a 12 - 21% enhancement on methane production from WS for all the examined scenarios, while the adjustment of the C/N was more clear when was combined with a steam explosion pretreatment step. Furthermore, all four different examined feedstocks (WS, SE, DDGS and RM) performed well as sole substrates in a batch AD system with preferable OLR of 12 g VS/L over that of 6 gVS/L. It was also found that the addition of nitrogen, either inorganic (NH_4Cl) or organic as a co-substrate, was more efficient towards the increase of gas production from the SE samples rather than from WS. This result could be attributed to the increased metabolic needs of the microorganisms fed with the easier degradable SE compared to WS. It was also found that when higher amounts of DDGS or RM were added to the system (50:50 w/w co-digestion scenario) higher methane yields were produced compared to the rest

of the examined digestion ratios. However, the addition of lower amounts of the two FBP was more beneficial towards the increase of the anaerobic biodegradability of WS and SE. Finally, according to the results of this study, it was proposed that the addition of 10% of either DDGS or RM on the AD of WS can offer similar gas yields to the ones produced after the digestion of solely SE. Based on the results of this study, it can be concluded that the addition of 10% of either DDGS or RM on the AD system operating with WS can enhance gas yields, in levels similar to those achieved steam-exploded straw. Further techno-economic evaluation and life cycle assessment (LCA) could determine the financial sustainability of commercial biomethane production based on these co-digestion strategies.

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6 General Discussion

The main aim of the thesis was to investigate the effect of steam explosion pretreatment on the biomethanation of wheat straw (WS) and to establish the optimum operational conditions for the digestion of this lignocellulosic residue in AD systems. Up to now, most studies that have evaluated steam explosion pretreatment of WS in AD systems have been conducted on a BMP digestion mode (Bauer et al., 2009; Theuretzbacher et al., 2015b). On the other hand, the literature lacks studies where the anaerobic biodegradability of steam-exploded WS (SE) is evaluated in a continuous digestions system. Furthermore, the literature also lacks studies examining the microbiology of AD systems operating with steam-exploded wheat straw (SE) as a substrate. A key target of the thesis was to cover these knowledge gaps with experiments designed both on a small, batch scale (BMPs) as well as on a continuous operation mode in AD reactors. An enhancement on the biogas yields offered by the AD of WS and depending on the selling prices of it can potentially render this feedstock more financially sustainable for full-scale biogas plants, compared to energy crops, as it would eliminate the cost of the cultivation of the later. Furthermore, due to the competition between energy crops and crops intended for human consumption for the available space in the arable land, a substitution of the first with WS as an AD feedstock can potentially have more social outcomes as the prices for food will be reduced due to its increased availability.

Apart from the enhancement of biogas production from WS, an additional target of the present PhD was to minimise the mechanical problems caused to AD systems due to the use of this residue as feedstock. This is the first time that research on the topic of anaerobic digestion is taking place at the University of Reading and so the whole

laboratory set-up had to be designed and built from scratch. The experiments of this PhD were conducted either on a continuous digestion mode or on a batch mode (BMP). The continuous CSTR system was used for a total experimental period of more than 500 days. During this period and due to the pressure that WS cause in the system, improvements had to be applied in the design of the bioreactor including the replacement of the plastic stirrers with alternatives made of stainless steel and decreased paddle size. Furthermore, during the first CSTR experiment, NaOH was used in order to capture the CO_2 and then measure only the desirable CH_4. The fast biogas production, especially in the case of the SE feedstock, created problems as the reaction of NaOH with the CO_2 produced crystals that accumulated and blocked the gas tubes. As a consequence, the pressure inside the bioreactors was continuously building up increasing the risk of the operation. In order to avoid this, a portable gas analyser (Biogas 5000, Geotech USA) was used to replace the NaOH solution and to characterise the quality of the produced biogas. The technical issues that WS causes when used as a feedstock in an AD system are not limited to the lab-scale digesters but also potentially occurring on a full-scale digestion system. As a consequence, the outcomes from the present PhD can be used not only towards the biological enhancement of the AD of WS but also towards the elimination of the mechanical problems even on a commercial scale digester.

Although steam explosion pretreatment was the main focus of this project, a slightly different hydrothermal pretreatment, autoclaving, was preliminarily assessed for its effect on WS digestibility and biogas production. One of the key findings in these preliminary studies was that the composition of WS did not change greatly after the application of the autoclave pretreatment. However, based on the SEM analysis, structural changes were noted as the surface of the steam-exploded samples was

rougher and more disordered compared to WS. It was assumed that these structural alternations highly contributed to the increase of the biomethane yields from WS.

Furthermore, based on modelling simulations presented in chapter two, the relationship between the OLR and the hydrolysis coefficient was not linear and followed a parabolic trend, indicating a strong effect of the substrate concentration in the substrate degradation kinetics. Practically, this means that the autoclave pretreatment at the higher and lower OLR offered higher improvement in the gas yields compared to the middle range OLR. It is possible that after the use of the pretreatment higher OLR can be successfully applied which subsequently means that the produced gas yields can also be increased. Finally, the addition of the liquid by-product of the autoclave pretreatment was found to have a potential inhibitory effect on the AD, especially when a low OLR was applied. A potential increase in the solubility of the easily degradable components of WS, after the autoclave pretreatment, would increase their presence in the liquid by-product of the pretreatment. Rapid production of VFAs due to the high availability of these components to the hydrolytic and acidogenic microorganisms of AD could potentially explain the reduced biomethanation in the cases where the whole sludge produced by the pretreatment was digested. The lower availability of the AD microorganisms in the cases when higher dilution (lower OLR) of the inoculum was applied can explain the increased inhibition effect compared to the cases where a higher OLR was applied.

In the third chapter of this thesis the effect of steam explosion pretreatment on biogas production from WS, both in BMPs and in continuous mode, was evaluated. Similar to the results from the autoclave pretreatment conducted at 140 °C, the steam explosion, which occurred between 140 °C and 175 °C, was found to affect mostly the structure of straw rather than its chemical composition. According to the literature, the above

temperatures resulted in a severity factor too low for causing significant solubilisation of hemicellulose while cellulose will require even higher energy according to Garrote et al. (1999).

During the BMP experiment, thirteen steam explosion pretreated WS samples, with differences in the severity factor (SF) of the different pretreatments, were tested against the initial residue and another two different batches of WS. The average improvement in the gas production reached values close to 20% (v/v) compared to the gas yields from the untreated WS samples. However, no clear correlation was found between the severity factor of the pretreatment (ranged between 2.9 and 3.35) and the enhancement of the biomethane production and as a consequence, no clear conclusion can be made towards the optimisation of the pretreatment conditions.

SE was also evaluated as AD feedstock in a continuous digestion mode. The experimental period was divided into two sub-periods based on the OLR that was applied to the reactors (2 and 5 g VS/L day^{-1}). The operation of the reactors was generally stable with some short periods of instability that occurred mostly due to mechanical problems on the operation of the reactors. With respect to the gas yields, for the periods when all reactors were operating under steady-state, the steam explosion pretreatment was found to increase the methane yields on an average level of 20% (v/v), similar to the results from the BMP trials. Biologically, the AD system seemed to adapt very fast to the increase of the OLR as the gas yields were restored to their previous values shortly after the change. In addition, a shift, regarding the different microbial abundances, was observed for all four reactors after the increase of the OLR to 5 g VS/L day^{-1}, while the microbiological analyses revealed that the steam explosion pretreatment had an insignificant effect on the bacterial population inside the bioreactors. In more details, the order of *Bacteroidales* and mainly the family of

Porphyromonadaceae seemed to overtake and dominate the bioreactors after the increase of the OLR to 5 kg VS/m^3. On the other hand, the most abundant microbial order, when the system worked under an OLR of 2 kg VS/m^3, were *Clostridiales* and specifically the families of *Caldicoprobacteraceae* and *Clostridiaceae*.

In the next chapter of the thesis, a comparison between the steam explosion and mechanical pretreatment on WS biomethanation was pursued. In the past, different pretreatment choices have been examined as a method to enhance the biogas production form non-easily degradable feedstock. The already examined pretreatment included biological, chemical, mechanical and thermal techniques. The main reason for choosing to examine and compare the steam explosion and the mechanical pretreatment, is the high cost for the biological methods (Hosseini Koupaie et al., 2019) and the need for managing potential by-products derived form a chemical pretreatment. Both the steam explosion and the mechanical pretreatment have been studied for their impact on the anaerobic biodegradability of WS (Theuretzbacher et al., 2015b; Tsapekos et al., 2018). However, their combination prior to the AD of this feedstock and other lignocellulosic residues has barely been examined in the literature.

Based on the BMP trial's results, both pretreatments can potentially increase the produced gas yields, while steam explosion seemed to slightly outperform the mechanical pretreatment. It was also noticed that when both methods were applied to WS, the gas yields were not significantly increased any further compared to the yields offered from the steam-exploded samples.

In order to validate these findings, a CSTR experiment was also designed and ran for 12 months. WS mostly due to its high moisture content proved to be very resistant in the mechanical pretreatment. In order to avoid the energy-intensive and commercial unstainable process of drying the feedstock prior to the mechanical pretreatment, a

portable straw chopping machine was built and used for decreasing the particle sizes of this feedstock before it was fed in the CSTR system.

During the periods of stable CSTR operation, when low fluctuation on the gas yields was reported, increased biomethanation for the SE was noted compared to both the untreated and the mechanically pretreated WS. However, the steam explosion pretreatment also resulted in increased instability of the AD system. More specifically, the digesters fed with steam-exploded feedstock showed a lower buffering capacity, a higher concentration of VFAs and decreased microbial diversity compared to the rest of the bioreactors. It is possible that the increased availability of the anaerobic biodegradable components of WS, due to the steam explosion pretreatment, caused a rapid production of VFAs which subsequently resulted in the increased instability of the system.

Furthermore, the microbiological analysis showed that the steam explosion pretreatment had a more significant effect on the ecology of the system compared to the mechanical pretreatment. For the two digesters fed with SE or steam-exploded and chopped straw (SEC) the most abundant bacteria family found was *Porphyromonadaceae* with a presence up to 70% of the total microbial population. In the past, this family has been reported as the most dominant in processes where easily degradable lignocellulosic feedstock, such as maize silage, is digested and its metabolism has been connected to the utilisation of VFAs (Poszytek et al., 2017). Higher production of VFAs from the steam-exploded samples compared to WS can explain the increased presence of this microbial family. On the other hand, the presence of this family in the digesters fed with either WS or chopped straw (WSC) was significantly lower. Furthermore, an unidentified species from the family of *Clostridiales* (*Clostridiales;__;__),* was found to be the second most abundant family

in reactors fed with SE and SEC but at the same time was only found in low concentrations (up to 10% of the total microbial biomass) in the reactors digesting WS or WSC. It is also worth mentioning that for the two digesters fed with non-steam exploded straw, no microbial family found to be more dominant compare to the rest of the families. When the archaeal populations were examined, it was found that an uncharacterised archaebacterium from the family of *Crenarchaeota* (*Crenarchaeota; c__MCG;o__pGrfC26;f__;g_*) was the most dominant species for all bioreactors. The family of *Methanosarcinaceae* (genus of *Methanosarcina* sp.) was also found in increased concentrations in the digester fed with SE especially in the cases when the VFAs were increased. Studies in the past already stated that this type of archaebacteria can adapt to difficult environmental conditions such as the high concentration of salt, high presence of ammonia and other material that could potentially inhibit the AD process (De Vrieze et al., 2012).

These results were not in total agreement with the data presented in the third chapter of this thesis where it was shown that the bioreactors fed with steam-exploded straw did not have significant differences in their microbial populations compared to the digesters fed with WS. This difference can be attributed to the lower hydraulic retention time (HRT 30 days) applied in the experiment of chapter four compared to chapter three (HRT higher than 100 days for the OLR of 2 kg VS/m^3). It is possible that due to the decreased HRT applied the reproduction rate for the microorganisms of the system was affected and this caused the changes in the microbial population of the bioreactors (Elsayed et al., 2016).

Finally, the last research chapter of this thesis examined the effect of WS steam explosion pretreatment along with the addition of a nitrogen source aiming at a balanced C/N in the AD system. At first, inorganic nitrogen (NH_4Cl) was used to balance the

C/N to 30 (w/w), which according to the literature is within the optimal range for AD systems (Estevez et al., 2012). Based on the gas measurements, SE not only offered higher yields compared to WS, as similarly shown in the previous chapters, but its biomethanation was further improved when the external nitrogen source was introduced to the system.

Next, two different agri-industrial by-products with a high concentration in proteins, distiller's grains with solubles (DDGS) and rapeseed meal (RM), were used as a co-feedstock for both WS and SE, aiming at balancing the C/N. The co-digestion scenarios seemed to be more beneficial for the steam-exploded straw rather than the WS. These results were attributed to the increased metabolic needs of the AD microorganisms in the cases when SE was digested due to the increased availability of the feedstock's degradable components. In more details, increased availability of the feedstock resulted in an increased need for nitrogen and since the later was provided from the co-feedstocks, the biomethanation of WS was subsequently enhanced.

Finally, it was also found that replacement of only 10% (w/w) of the untreated WS with either DDGS or RM can offer an increase in the gas yields comparable to the enhancement that the steam explosion pretreatment can offer. These results can be attributed to the high gas yield from the two agri-industrial by-products as well as the balanced C/N ratio that they offered in the digestion of straw. According to this and depending on the availability and the price of these two agro-industrial by-products, it is possible that a co-digestion strategy could enhance the financial sustainability for the digestion of WS in AD systems (and possibly other lignocellulosic waste with similar chemical composition to WS), without the need of implementing an additional pretreatment step.

In conclusion, the optimisation of the anaerobic digestion conditions for the SE has not been examined in-depth in the past. Regarding the OLR of the system, BMP experiments show that an increased OLR might be preferable when the steam-exploded straw is the main AD feedstock. It is likely that an OLR of 5 gVS/L day^{-1} can be sustainable for the continuous digestion of WS. However, an increased OLR can potentially be linked with a lower HRT in the digester which was also proved in the present thesis to be unfavourable for the AD of steam-exploded wheat straw. The consequences of the decreased HRT included a low buffer capacity, a reduced pH and an accumulation of VFAs. The effect of the HRT on the digestion of steam-exploded WS is also linked to the microbial diversity of AD systems. More specifically, the reduction in the HRT of the system also boosted a shift in the microbial populations for both the reactors digesting untreated WS and those digesting steam-exploded straw. It is also worth mentioning that the alternation in the population of bacteria was more profound compared to the changes reported on the population of archaebacteria colonizing the anaerobic bioreactors. However, despite its increased instability, the AD system fed with steam-exploded feedstock seemed to be able to recover after a partial re-inoculation and a balanced supply of buffer solution. This thesis also showed that a balanced C/N is required for exploiting the maximum possible energy from WS regardless of the use or not of the pretreatment.

6.1 Future work

WS is generally considered as a challenging and difficult digestible feedstock for the process of AD, nevertheless, it's high biogas potential which renders this residue very promising for feedstock for full-scale biogas plants. As mentioned above, for the next step of this thesis, the steam explosion pretreatment of WS needs to be tested on a full-scale digestion system in order to validate the effectiveness and the sustainability of its

commercialisation. Operational parameters such as the OLR, the HRT, the temperature, the production and accumulation of VFA, the pH and the creation of floating layers of straw inside the digesters might affect the digestion of SE. Also, on long term digestion, a depletion of the necessary, for the microorganisms of AD, micronutrients might occur due to their limited availability in WS (Kainthola et al., 2019). In the present study, a commercially available solution was used in order to balance these potential deficiencies. However, further research is required in that direction in order to validate the nutritional needs of both WS and SE and establish the optimal rate of supplementation.

Furthermore, according to the results of the present study, the continuous digestion of both WS and SE was efficient at an OLR of 5 kg VS/m^3 while an OLR of 12 gVS/l was also acceptable when a BMP digestion was evaluated A gradual further increase of the OLR along with an HRT higher than 30 days must be tested in the future in order to identify potential enhancement of biogas production from WS. In that direction, a larger digestion system (commercial full-scale digester) will be required in order to eliminate the mechanical limitations that the particle size of wheat straw offers to the system. However, limitations, including the negative effect that the decreased HRT had in the digestion SE, needs to be taken into consideration when these experiments are designed. Also, further work is required on identifying the role and the interactions between the different microbial populations inside the reactors digesting either untreated or steam-exploded WS. A better understanding of the microbiology of the AD of WS can also contribute to the enhancement of biogas production from this residue on a commercial scale. A bio-augmentation of the digester with specific microorganisms have already proposed in the past as a method to enhance biogas production (Lins et al., 2014; Strang et al., 2017). A combination between the steam explosion pretreatment and the addition

of specific microbial communities can potentially increase the biogas yields and also provide the necessary stability to the system in order to overcome inhibitory effects, such as the increased presence and accumulation of VFAs.

Finally, prior to the commercialisation of this technology, there is a need to perform a techno-economic assessment (TEA) of steam explosion pretreatment of WS in AD systems while a comparison with the mechanical pretreatment can also be evaluated. Although the current study demonstrated that SE can offer up to 20% higher biogas production, the translation of this strategy on a commercial scale necessitates the assessment of capital and operational costs as well as that of return of investment. Also, in order to justify that the increase in the energy production succeeded after the application of the steam explosion pretreatment is higher than the energy required for running the economizer, parameters such as energy consumption of the equipment and the energy spend for the transportation and the storage of the feedstock need to be included in the calculations. Despite the fact that the evaluation of energy consumption of the economizer was not in the scope of this work, this has been included in the future goals of the project. In the same context, TEA could also evaluate the incorporation of inorganic nitrogen and/or co-substrates as suggested in the current thesis (NH_4Cl, DDGS and rapeseed meal) as a means of increasing biomethanation yields. More specifically, the additional energy recovered from WS after balancing its C/N needs to be compared with the cost for the purchase and storage of the above additives and co-substrates.

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