Effects of biochar parent material and microbial pre-loading in biochar-amended high-solids anaerobic digestion

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Abstract

This study characterises the effect of biochar (pyrolysed biomass) produced from wood pellets, wheat straw and sheep manure on high-solids anaerobic digestion (HSAD) of poultry litter. Also, pre-loading biochar with microorganisms before addition to HSADs was investigated. The addition of wood pellet biochar provides a 32% increase to the methane yield compared with control digesters. The addition of biochar produced from either wheat straw or sheep manure has detrimental effects on digester performance compared with controls. The addition of wood pellet biochar pre-loaded by placing it in a high-solids digester for 90 days provides a 69% increase in the total methane yield, 44% increase in the peak daily methane yield and a 33% reduction in the lag time compared with controls. This study highlighted a need for careful selection of parent material for biochar production and, for the first time, the opportunities to re-use wood pellet biochar for further improvements.

Keywords: Anaerobic digestion, Biochar, Poultry litter, Gasifier, Biogas

1. Introduction

Anaerobic digestion is the biological degradation of organic material by a diverse variety of microorganisms in an oxygen-free environment. The process produces methane-containing biogas which can be used for heat and electricity generation and could be used as a transport fuel. A wide variety of feed-

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stocks can be used for anaerobic digestion, and the total solids (TS), or dry weight, of the feedstock can determine the digester design. Solid wastes such as manure mixed with bedding material, which can have TS contents of 20–60% are suggested to be more suitable for high-solids anaerobic digesters (Riggio et al., 2017). The TS of content in the bulk sludge of high-solids anaerobic digesters (HSAD) is around 20% compared with 5-10% for conventional stirred tank low-solids digesters (LSAD). This is because of lower water requirements (Li et al., 2018) and bedding material, such as straw or wood shavings, causing clogging problems in the conventional stirred tank low-solids digesters (Chanakya et al., 1997).

A significant benefit of high solids digesters is their smaller volumes. As a result, a high-solids digester can have up to three times higher volumetric efficiency (volume methane produced per volume of digester) compared with a low-solids digester (Li et al., 2013). Draw-backs of high solids digesters, caused by lower water requirements, are an excessive concentration of substances such as ammonia, sulphides, light metal ions and heavy metals which can inhibit the anaerobic digestion process (Chen et al., 2008). In addition, a lower water content results in lower rates of hydrolysis of solid organic material into soluble products which can be metabolised by microorganisms (Batstone and Jensen, 2011) and lower rates of mass transfer of these soluble products within the digester (Bollon et al., 2013)). As a result of these two factors, high-solids anaerobic digestion of manures generally has lower total methane yields and lower methane production rates than low-solids anaerobic digestion of the same feedstock (Li et al., 2013; Tait et al., 2009). There exists a need for a low-cost method to improve high-solids digester performance.

The addition of a wide variety of conductive materials have been shown to improve anaerobic digester performance. The materials include biochar (Cruz Viggi et al., 2017; Pan et al., 2019; Zhao et al., 2016), activated carbon (Park et al., 2018) and magnetite (Cruz Viggi et al., 2014). The benefits are suggested to occur via the stimulation of direct interspecies electron transfer (DIET) between bacteria and methanogens both attached to the biochar (Lei et al., 2019). DIET provides an additional pathway to the standard hydrogen/formate interspecies transfer of electrons (Holmes and Smith, 2016). The stimulation of DIET

eliminates the rate limiting step of diffusion of soluble electron carriers, hydrogen and formate (Cruz Viggi et al., 2014) and thereby improving methane production performance. This may be one way to improve rates of methane production in high-solids digesters.

The use of biochar, a solid residue produced from pyrolysis of biomass, is particularly attractive for use in digesters located in rural and resource-constrained communities. Biochar can be produced from a variety of robust technologies such as earth pits, rotary kilns, furnaces and gasifiers. Also, it can be produced from a variety of parent materials such as wood, manure or crop residues. While this may be an advantage, biochar properties vary due to production process and parent material (Enders et al., 2012) which creates a need for an understanding of how biochar produced from different parent materials can have varying effects.

Biochar produced from wood (Cruz Viggi et al., 2017; Fagbohungbe et al., 2016), agricultural wastes such as rice husk (Fagbohungbe et al., 2016), wheat bran (Cruz Viggi et al., 2017), wheat straw (Shen and Zhu, 2016) or manures (Jang et al., 2017; Pan et al., 2019; Wang et al., 2017) have all shown to improve digester performance. In addition, the use of biochar produced from different parent materials digesting the same feedstock has been the subject of previous investigations (Fagbohungbe et al., 2016; Pan et al., 2019). Surprisingly, there are only a few studies that report detrimental effects of biochar addition to anaerobic digesters. At high dosages, biochar produced from walnut shells (Linville et al., 2017), cardboard (Li et al., 2019), cow manure (Sun et al., 2019) and corn stover amended with iron (Zhang et al., 2019) have detrimental effects on performance. Excessive concentrations of light metal ions (such as sodium, potassium, magnesium, calcium and aluminium) within the biochar was suggested in one study where poor performance was observed (Linville et al., 2017). A possible reason why few negative results of biochar addition have been reported could be the focus on biochar addition to low-solids digesters as opposed to high-solids digesters, which is the focus of this study. Diluting the feedstock with water is a common method to avoid excessive concentrations of inhibitors (Chen et al., 2008), however, substantial dilution with water negates the advantages of high-solids digestion. A deeper understanding of biochar properties that improve or negatively affect high-solids digesters will allow for the selection

of suitable parent materials for biochar production.

The population of methane-generating microorganisms attached to biochar varies over time (Kuroda et al., 1988; Lü et al., 2016), furthermore, the formation of a complex microbial community on a solid support can take several days (Wolferen et al., 2018). The use of biochar already loaded with microorganisms may further increase methane production rates compared with the addition of raw biochar. Biochar pre-loaded with microorganisms could simply be biochar recovered from a previous batch, in the case of high-solids digesters, or from the effluent from a low-solids digester. From a practical perspective, the ability to reuse biochar could decrease costs (both time and money) associated with the constant production of biochar. Pre-loading of biomass that has not been pyrolysed has been shown to increase methane production rates (Zainab et al., 2019), however, there is little understanding of how pre-loaded biochar will affect anaerobic digestion performance. Initial investigations are needed to determine the long-term sustainability of biochar addition in digesters.

This study aims to characterise the effect of different types of biochar produced from a gasifier on high-solids anaerobic digestion of manure. Poultry litter was selected as the manure. The objectives are; (i) to characterise the biochars produced from three different types of biomass; wood, wheat straw and sheep manure, (ii) determine the effects of adding these biochars on methane yield and production rates and (iii) to determine the effect of pre-loading biochar with microorganisms on the methane production rate and yield by using two methods for microbial loading.

2. Methods

2.1. Methane production assay

The poultry litter was batch digested in 500 ml sealed glass bottles. The volume of biogas produced was measured by the displacement of saturated sodium chloride solution. The volume of biogas produced was corrected to dry gas at 0°C. The digesters were kept at 37°C. Each digester was mixed for 10 seconds, once per day, five times per week. Each testing scenario was conducted in triplicate to account for potential biological variation.

The feedstock, poultry litter, was sourced from a broiler-chicken farm in South Australia, which uses pine wood-shavings as the bedding material. The source of methane-generating microorganisms (the inoculant) was centrifuged anaerobic digester effluent from a wastewater treatment facility (SA Water, South Australia). The inoculant was maintained under anaerobic conditions for three days at 37°C to reduce its residual methane production potential, while maintaining an active microbial population. The volatile-solids based feedstock to inoculant (F/I) ratio was 2 in digesters with both raw and pre-loaded biochar. The total solids content of the digesters was adjusted to 20% using Milli-Q water. The calculation of total solids did not include the total solids content of the biochar. The head-space in each digester was purged with high-purity nitrogen gas to produce anaerobic conditions.

2.2. Biochar production

The parent materials used for biochar production were; (i) wood pellets, (ii) wheat straw and (iii) sheep manure. The wood pellets were commercially-available and produced from timber waste from multiple mills around Australia. The wheat straw was commercially available cut straw harvested in South Australia. Sheep manure was collected from grazing animals in South Australia and no bedding material was included. The biochar from these feedstocks was produced in an auto-thermal top-lit updraft (TLUD) gasifier. The primary air input was constant for each feedstock. As a result of the auto-thermal nature of the gasification reaction, the peak temperature inside the gasifier varied depending on the feedstock. The peak temperature inside the gasifier for wood pellets, wheat straw and sheep manure were 770°C, 680°C and 720°C, respectively. The feedstocks had an average residence time of 2.5 hours. Wood pellet biochar was cylindrical, approximately 16 mm in length and 4 mm diameter. The sheep manure biochar varied between 5–15 mm in diameter and 5–10 mm in height. The wheat straw biochar was approximately 5–10 mm in length, 3–4 mm width and 0.8 mm in height.

Wood pellet biochar was selected for pre-loading with microorganisms. Two digestion times and total solids regimes were used for these digesters; (i) wood pellet biochar placed in a low-solids digester (total solids = 5%) for 30 days, and (ii) wood pellet biochar placed in a high-solids digester (total solids

= 20%) for 90 days. The same feedstocks and inoculant (poultry litter and wastewater treatment plant sludge) were used for the pre-loading digesters. The dry mass of biochar was equal to the dry mass of poultry litter in each digester. This dosage is within the range used in other work (Fagbohungbe et al., 2016; Li et al., 2019). At the end of the digestion period, the biochar was removed from the digesters. The biochar was separated from the digestate using tweezers and loosely attached sludge was removed from the biochar by rinsing with Milli-Q water.

2.3. Biogas analysis

Biogas samples were sampled periodically by extracting the headspace into gas-tight 10 ml glass syringes. The composition of CH_4, CO_2 and H_2 in the gas was determined by a gas chromatograph with a thermal conductivity detector (Agilent, 490 MicroGC). Additional details on the gas chromatography method have been reported previously (Indren et al., 2020).

2.4. Chemical analysis

The total solids content of the biochar, feedstock, inoculant and bulk sludge was determined by drying at 105°C in an oven (Clesceri et al., 1999). The volatile solids content was determined by ashing the materials at 550°C (Clesceri et al., 1999) in a thermogravimetric analyser (Mettler Toledo, TGA-DSC2). Aqueous samples of solid materials (feedstock, inoculant and bulk sludge) were produced by diluting 5 g of the material in 20 ml of Milli-Q water, mixing for 20 minutes and then centrifuging at 2000G for 10 minutes. The pH of the aqueous supernatant was measured using a pH probe (Mettler Toledo, InLab Expert Pro®). Total alkalinity was determined by titration against 0.1 N H_2SO_4 (Clesceri et al., 1999). Total ammonia-nitrogen was determined colorimetrically using the salicylate method (Forster, 1995). The free ammonia-nitrogen concentration was calculated according to the equation given by Hansen et al. (1998).

The volatile fatty acid composition of the bulk sludge at the end of the 90-day digestion period was measured using a Perkin Elmer SQ8 Gas Chromatograph-Mass Spectrometer (GC-MS). Aqueous samples were prepared using the same extraction procedure used for pH, TAN and TA measurements. A

1 ml aliqout of the solution was was acidified using 100 µl of phosphoric acid to reach a pH <2. This aliquot was centrifuged for 10 minutes at 13400 rpm in a benchtop centrifuge (Eppendorf, Minispin®) and passed through a 0.45 µm syringe filter (Sartorius, Minisart® NML). Compound separation was undertaken using a COL-Elite-FFAP capillary column (Perkin Elmer, 30 m × 0.25 mm ID × 0.32 µm phase thickness) with helium carrier gas at a flow of 2 ml/ min. One microlitre of the samples were injected in split mode (50:1) with injection temperatures of 250°C. The oven temperature was held at 50°C for one minute, before a 10°C/minute ramp to 240°C and a final hold of 5 minutes. The mass spectrometer scanned from m/z 50–400 at approximately 3 three scans per second. Data interpretation was undertaken using Perkin Elmer TurboMass 6.0 software with a comparison of compound spectra to the NIST14 Spectral Library Database. A seven-point calibration curve and reproducibility validation for C2-C7 volatile fatty acids was constructed using a certified volatile free acid mix (Supelco, CRM46975). A three-point calibration check was analysed with each sample batch. The total VFA concentration was calculated by summing the concentration of C2-C7 volatile fatty acids.

2.5. Biochar characterisation

The composition of carbon, hydrogen and nitrogen of the biochars was determined by combustion using a Perkin Elmer, 2400 Series II Elemental Analyser. Total oxygen content of the biochar was derived by subtraction according Equation 1.

$$O\,(\%\ of\ TS) = 100 - ash\,(\%\ of\ TS) - C\,(\%\ of\ TS) - N\,(\%\ of\ TS) - H\,(\%\ of\ TS). \qquad (1)$$

Inductively coupled plasma (ICP-OES) analysis (Agilent Technologies 5100) was conducted on the biochars for analysis of trace elements in the biochar (CSIRO, South Australia). Before ICP-OES analysis, the biochar was dissolved into a solution using the microwave digestion method with a mixture of nitric acid and hydrochloric acid (reverse aqua regia). The pH of the water-extractable fraction of biochar was determined following a procedure outlined by Singh et al. (2017). One gram of biochar was mixed with 20 ml of deionized water and then agitated on an orbital shaker table for 1 hour and left

to rest for 30 minutes. The slurry was continually mixed with a stir bar while pH was measured. The total alkalinity of the biochars was determined by the rapid titration method (Singh et al., 2017). Before analysis, 0.5 g of biochar was placed in 1 M HCl, shaken for 2 hours and left to stand overnight. Titration was conducted using 0.5 M NaOH until a neutral pH (7.0) was reached. Bulk density of the biochars were determined by weighing the amount of material loaded (without packing) into a cylinder (diameter 73 mm, height 48 mm).

2.5.1. Microbial population analysis

The population of methane-generating microorganisms in the inoculant, digester bulk sludge and on each type of biochar were analysed. Prior to DNA extraction, the biochar was washed three times in 1 ml of phosphate-buffered saline (PBS) to remove loosely attached sludge. The biochar was then crushed using a mortar and pestle. To ensure biofilms containing microorganisms were broken apart, the crushed biochar (0.25 g) was placed in a tube with 0.5 ml of PBS and sonicated in a bench-top ultrasonic cleaner (Soniclean 160HD) for 2 minutes using 15-second pulses. After sonication, the supernatant and crushed biochar were placed into a powerbead tube from the PowerSoil DNA isolation kit (Quiagen, Germany). DNA was extracted following the kit instructions. No sonication or crushing was conducted for the inoculant or bulk sludge samples. The quantity of DNA extracted was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Quantitative polymerase chain reaction (qPCR) was conducted using an iCycler (Bio-Rad Laboratories, Hercules, CA) to determine the abundance of *Methanosaetaceae*, *Methanosarcinaceae*, *Methanobacteriales* and *Methanomicrobiales* in the inoculant, bulk sludge and attached to the biochar. The primer sets were developed by Yu et al. (2005). The qPCR procedure followed a two-step amplification procedure described in a previous study (Indren et al., 2020).

A scanning electron microscope (XL30, Philips) was used to investigate the attachment of microorganisms onto each type of biochar. The biochar was prepared for analysis by first removing any residual sludge by washing three times with 1 ml of PBS. Details on the sample preparation method have been

reported previously (Indren et al., 2020).

2.6. Data analysis

Statistical analysis was conducted using R (version 3.5.0) and included one-way analysis of variance (ANOVA) with a significance value of 0.05. The Tukey post-hoc test, with a significance value of 0.05, was used for a comparison of mean values between each scenario. The modified Gompertz equation modelled using the Grofit package of R-project software (version 3.5.0) was used to estimate the potential methane production, maximum methane production rate and methane production lag time. In one test (the control), one of the three replicate digesters was lost due to cracking of the airtight sealing.

3. Results and discussion

3.1. Biochar properties

Table 1 and Table 2 show properties of biochar produced from wood pellets, wheat straw and sheep manure. The parent material affects both the chemical and physical composition of the biochar. Key differences in the chemical composition between the three types of biochar include a lower ash content (0.3 ± 0.4% of TS) in wood pellet biochar compared with the wheat straw biochar (14 ± 2.0% of TS) and sheep manure (58 ± 0.2% of TS). As a result, wood pellet biochar had the lowest concentrations of calcium (Ca), potassium (K), sulphur (S), magnesium (Mg) and sodium (Na). These elements, at excessive concentrations, have been shown to have inhibitory effects in anaerobic digestion (Chen et al., 2008; Mccarty and Mckinney, 1961). Furthermore, the concentration of alkaline elements, K, Na, Ca, and Mg, are lower in the wood pellet biochar. This is likely the cause of its lower total alkalinity (7.3 ± 2.1 g-$CaCO_{3eq}$/kg) and will lower its ability to prevent acidification due to the build-up of volatile fatty acids which commonly occurs in ammonia-stressed digesters. A key physical feature of the wheat straw biochar is its four times lower bulk density than the other two biochars. As a result, the inclusion of wheat straw biochar will increase the digester working volume and lower its volumetric efficiency.

Figure 1 shows the concentration of methane-generating microorganisms (methanogens) and total microorganisms on the two types of pre-loaded wood pellet biochar. The two types of pre-loaded biochar are (i) wood pellet biochar taken from a low solids digester after 30 days (WP30L), and (ii) from a high-solids digester after 90 days (WP90H). The targeted methanogens were the strictly acetate-consuming *Methanosaetaceae* family, the acetate or hydrogen-consuming *Methanosarcinaceae* family, and the strictly hydrogen-consuming orders *Methanobacteriales* and *Methanomicrobiales*. The figure shows the time for pre-loading and the total solids content of the pre-loading environment affect the proportion of microorganisms that attach to the biochar. There is approximately 6 times higher concentration of total microorganisms on WP90H compared with WP30L. Figure 1 also shows there is a similar concentration of the dominant methanogen, *Methanosaetaceae*, on WP30L and WP90H. *Methanosaetaceae* is the dominant methanogen despite its lower resistance to ammonia stress compared with *Methanosarcinaceae* and the hydrogen-consuming methanogens (Angelidaki and Ahring, 1993; Calli et al., 2005).

3.2. Effect of biochar type on methane generation

Figure 2 shows the cumulative methane yield over 90 days in high-solids digesters processing poultry litter with biochar produced from three parent materials, two types of pre-loaded wood pellet biochar and digesters without biochar (control). The line represents the mean value, the error bars show the standard deviation and markers show the range of values between three biological replicates. The effect on cumulative yield varies depending on the type of biochar added. The addition of wood pellet biochar increases the mean cumulative methane yield by 32% (66 ml CH_4/g-VS) compared with the controls (50 ml CH_4/g-VS). The addition of wheat straw biochar or sheep manure biochar had no substantial effect on the mean cumulative methane yield. A large variation in the methane production rates between replicates of digesters containing wheat straw biochar were observed. Possible causes for the variation are discussed in Section 3.3

The two types of pre-loaded wood pellet biochar increased the mean cumulative methane yield compared with the control, however, the effect varied depending on the method of biochar pre-loading. The

addition of WP90H increased the mean cumulative methane yield to 87 ml CH_4/g-VS which was a 69% increase ($p<0.05$) compared with the controls. By comparison, the addition of WP30L increases the mean cumulative yield by just 22% to 61 ml CH_4/g-VS. This increase caused by the addition of WP30L was not statistically significant ($p> 0.05$). Also, the percentage increase in yield was smaller than the 32% yield increase caused by the addition of wood pellet biochar not pre-loaded with microorganisms. Possible explanations for the differences between biochar pre-loaded in the low and high-solids digesters are discussed in Section 3.6.

Figure 3 shows the daily methane yield over the 90 day digestion period for digesters processing poultry litter with biochar produced from three parent materials, two types of pre-loaded wood pellet biochar and without biochar (control). The line represents the mean, the error bars show the standard deviation and the markers show the range of values. The parent material used for biochar production had substantial impacts on the daily methane production rate. The addition of wheat straw biochar or sheep manure biochar resulted in the peak daily methane yield occurring later (day 63) compared with the controls (day 46). The addition of wood pellet biochar caused no significant change to the daily methane yield or the day at which it occurs.

The effects of pre-loaded wood pellet biochar on the daily methane yield varied depending on the pre-loading method. There was a 45% increase ($p<0.05$) in the mean peak daily yield due to the addition of WP90H (2.6 ml CH_4/g-VS/day) compared with the controls (1.8 ml CH_4/g-VS/day). Also, the peak daily day occurs on day 35, 11 days earlier than in the controls. The addition of WP30L, similar to wood pellet biochar that was not pre-loaded with microorganisms, had no significant effect on the daily methane yield or the day at which the peak daily yield occurs.

The changes to the daily methane yield due to biochar addition are also shown by changes to the time before substantial methane production begins (lag time). Table 3 shows the modelled Gompertz parameters; lag time, cumulative methane yield and maximum daily yield for digesters with each type of biochar. The addition of wood pellet biochar reduced the lag time by one day compared with controls (lag time of 25 days). Lag times substantially increased, to 39 days, for digesters with wheat straw

biochar and 46 days to digesters with sheep manure biochar. Due to a substantial variation in one of the three digesters containing wheat straw biochar, the Gompertz model did not produce a fit. The lag time for digesters with wheat straw biochar shown in Table 3 was calculated from only two biological replicates. This also resulted in a substantial range of predicted total methane yields for digesters with wheat straw biochar, and this value should be interpreted with caution. The addition of WP90H had the greatest beneficial impact on lag time and decrease the lag time by approximately 8 days (33%). The addition of WP30L decreased the lag time by 3 days.

3.3. Effect of biochar type on digester chemical conditions

To understand the mechanisms by which the addition biochar affects anaerobic digester performance, analysis of the of total ammonia-nitrogen, free ammonia-nitrogen, total alkalinity and total volatile fatty acids (VFA) was conducted on the bulk sludge. Table 4 shows the concentration of these substances in the digesters at the end of the 90 day digestion period. The high total and free ammonia-nitrogen concentration (6.4–8.1 g TAN/kg and 2.8–2.9 g FAN/kg) suggest that methane generation will be inhibited at the end of the 90 day digestion period. There is no statistically significant difference in either the total or free ammonia-nitrogen concentration between digesters with any type of biochar or the controls digesters. Digesters with pre-loaded biochar, WP30L and WP90H have slightly higher total ammonia-nitrogen concentrations compared with controls or digesters with raw wood pellet biochar which could be caused by residual ammonia associated with the biochar from the pre-loading step.

The total alkalinity of the bulk sludge from digesters with wheat straw biochar and sheep manure biochar (11.3–12.3 g-$CaCO_{3eq}$/kg) were not significantly higher than the bulk sludge of control digesters (12.8 g-$CaCO_{3eq}$/kg) or digesters with wood pellet biochar (14.7 ± 1.3 g-$CaCO_{3eq}$/kg). This occurs despite the three to four times higher alkalinity of wheat straw biochar and sheep manure biochar compared with wood pellet biochar. This may suggest the total ammonia-nitrogen concentration in the bulk sludge (6.4–8.1 g/kg) is the main driver of total alkalinity. As a result of the high total alkalinity in the bulk sludge, a high pH (8.8–8.9) is maintained despite VFA concentrations of up to 27.1 g/kg. This suggests

a high alkalinity biochar is not required to prevent acidification of high-solids anaerobic digesters.

A significant feature of Table 4 is the 66% lower total VFA concentration with the addition of WP90H. To understand changes to the total VFA concentration, the composition of the VFAs was analysed, with results shown in Figure 4. Acetate and propionate were the most abundant VFAs, except for digesters with WP90H, which had low propionate concentrations. Acetate and propionate accumulation is common for ammonia inhibited digesters (Tian et al., 2019) and their concentrations are reliable indicators of process performance (Boe et al., 2010). Acetate is directly used by the aceticlastic (acetate-cleaving) methanogenic families *Methanosaetaceae* and *Methanosarcinaceae* (Holmes and Smith, 2016). Inhibition of these aceticlastic methanogens will lead to high acetate concentrations.

Digesters with WP90H had a 92% lower concentration of propionate compared with the control digesters ($p<0.05$) and also showed complete degradation of isovalerate. The degradation of these two VFAs in digesters with WP90H may have lead to the increased methane yield. The degradation of propionate and isovalerate is thermodynamically unfavourable under standard conditions (de Bok et al., 2004; Stieb and Schink, 1986). However, propionate and isovalerate can be oxidized to acetate, bicarbonate, and hydrogen or formate through a syntrophic partnership between propionate/isovalerate-oxidizing bacteria and methanogens which consume the oxidation products (Barua et al., 2018; de Bok et al., 2004; Stieb and Schink, 1986). The concentrations of propionate or isovalerate were not affected by the addition of raw wood pellet or WP30L.

Only the poor performing digesters (digesters with wheat straw and sheep manure biochar) feature butyrate, a known indicator of process imbalance (Nakakubo et al., 2008). In addition, digesters with sheep manure biochar were the only digesters to have significant concentrations of hexanoate. This further suggests that methane production was inhibited in digesters with sheep manure biochar. Previous research has shown hexanoate (also referred to as caproate) production was enhanced via the reduction of butyrate to hexanoate facilitated by the addition of biochar produced from pine wood (Liu et al., 2017) in bioreactors where methane production was purposefully inhibited. To support this, the butyrate concentration was lower in digesters with sheep manure biochar compared with digesters with wheat

straw biochar.

The concentration of butyrate and acetate were highly variable in the three biological replicate digesters with wheat straw biochar. This may explain the variations in methane yield between these replicates. Valerate, isocaproate and heptanoate were not detected in any of the digesters (data not shown).

3.4. Effect of biochar type on biochar-microorganism interactions

Changes in methane production performance are expected to be evident by alterations in the digester microbial community. Figure 5 (A-D) and Figure 6 (A-D) show the concentration of DNA from key methanogens in the bulk sludge and attached to the biochar after 90 days. The dominant methanogen in both the bulk sludge and on the biochar was the acetate-cleaving *Methanosaetaceae* family. The concentration of *Methanosaetaceae* in the bulk sludge or on the biochar does not appear to be correlated with the methane yield. Despite the poor performance of digesters with wheat straw biochar, these digesters have the highest concentration of *Methanosaetaceae* in the bulk sludge and biochar. By comparison, the best-performing digesters (those with WP90H) have similar *Methanosaetaceae* concentrations in the bulk sludge compared with controls, and the concentration of *Methanosaetaceae* attached to WP90H is not significantly higher than the concentration on the other biochars.

The low concentration of the hydrogen-consuming methanogenic orders, *Methanobacteriales* and *Methanomicrobiales* in the bulk sludge is in agreement with previous studies of high-solids digesters operating under ammonia stress (Dai et al., 2016). Also, the concentration of *Methanobacteriales* on the biochar is higher than *Methanomicrobiales* for all types of biochar. The low concentration of *Methanosarcinaceae* in both the bulk sludge and attached to the biochar is likely due to its very low concentration in the inoculant.

Before methane generation, the anaerobic digestion process consists of three earlier steps; hydrolysis, acidogenesis and acetogenesis. The steps are facilitated by a diverse variety of bacteria. The concentration of total microbial DNA can indicate the total population of bacteria although this value will include DNA from other microorganisms. Figure 6 (E) shows the total DNA from all microorganisms

attached to each type of biochar at the end of the 90-day digestion period. All digesters with biochar have a higher total DNA concentration in the bulk sludge compared with the controls. However, due to the variation in the concentrations between biological replicates no statistically significant change could be determined.

The concentration of microorganisms shown in Figure 6 (E) suggests the parent material for biochar production affected the level of total microbial attachment onto the biochar. Sheep manure biochar had the lowest degree of microbial attachment (14 ng/µl/g-biochar). There was a higher degree of attachment onto wood pellet 31 ng/µl/g-biochar and wheat straw biochar (79 ng/µl/g-biochar). As expected, WP90H had a high degree of attachment (67 ng/µl/g-biochar). Attachment onto WP30L (22 ng/µl/g-biochar) was lower than raw wood pellet biochar.

It is possible the micro-structure of the biochar affected the degree of microbial attachment. Scanning electron microscopy analysis revealed the surface of the wood-pellet biochar is rough and has several cracks in the millimetre-size range. A rough surface will allow for microbial attachment and pores in the millimetre-size range would allow for microbial attachment inside the porous structure of the biochar. The surface of wheat-straw biochar is smooth with very few pores. Microbial attachment was not observed on this surface. The wheat straw biochar is constructed as layers of sheets. Pores along the edge of these layers could facilitate microbial attachment. Sheep manure biochar has pores in the 0.5 millimetre-size range but a fewer number than the wood pellet biochar. Also, the internal surface appears to be compacted and not as open as wood pellet biochar which would limit microbial attachment within sheep manure biochar.

The SEM imagery also showed shows a suspected biofilm on WP90H. That is a community of microorganisms, both rod-shaped and cocci, in this case, and embedded in an extracellular polymeric substance. No obvious biofilm formation was observed on the wheat straw manure or sheep manure biochar. Although in this observational analysis there can be biases from changes in the microbial load across each piece of biochar. The presence of biofilms and compact microbial communities on the biochar is significant as reduced distances between partnering microorganisms can decrease diffusion limitations of

intermediate products and soluble electron carriers, hydrogen and formate, allowing reactions to proceed at rates higher compared with reactions between microorganisms that exist as single cells (de Bok et al., 2004). Therefore, an increased degradation rate of intermediate substrates and higher methane production rates are expected.

3.5. Effects of biochar properties on digester performance

The results indicate the selection of parent material for biochar production and an understanding of beneficial and detrimental properties of the biochar produced is important. Wood pellet biochar was the only biochar to improve anaerobic digester performance. As an alternative to the reduced diffusion limitations due to attachment of microorganisms on the biochar, wood pellet biochar may have a greater ability to facilitate DIET than the other two biochars. This is evidenced by its molecular structure. In biochar with molar H/C and O/C ratios lower than 0.35 and 0.09, respectively, graphitic carbon structures emerge (Sun et al., 2017). This graphitic structure formed by pyrolysis temperatures greater than 700°C has a significantly lower resistance to the transfer of electrons compared with the amorphous structure in biochar produced at lower temperatures (Sun et al., 2017). Only the wood pellet biochar has H/C and O/C molar ratios below these threshold values (H/C = 0.26 and O/C = 0.07, Table 2). This is in agreement with higher peak temperatures observed during wood pellet biochar production compared with the other two biochars. By using the DIET mechanism, *Methanosaetaceae* does not rely solely on acetate for its metabolism and is likely not as greatly affected by low diffusion rates of acetate expected in the bulk sludge of high-solids digesters (Bollon et al., 2013). It is likely a combination of decreased diffusion limitations of soluble intermediates as well as the facilitation of DIET resulted in increased methane production rates in digesters with wood pellet biochar.

Sheep manure biochar likely has a more graphitic carbon structure than wheat straw biochar as evidenced by its lower H/C and O/C ratio (sheep manure: H/C = 0.34, O/C = 0.12 and wheat straw: H/C = 0.40, O/C = 0.24, Table 2) and therefore would exhibit lower resistance to electrons transfer. While this did not result in greater methane yields in digesters with sheep manure biochar, this may explain

the lower concentration of butyrate and the higher concentration of hexanoate (Figure 4) in digesters with sheep manure biochar compared with digesters with wheat straw biochar as hexanoate can be produced from butyrate via DIET (Liu et al., 2017).

It is possible the poor methane production performance of digesters with wheat straw and sheep manure biochar occurs due to inorganic elements (such as Ca, Mg, S, Na or K) in the biochar as observed with calcium and magnesium rich walnut shell biochar (Linville et al., 2017). The presence of butyrate, an indicator of process imbalance, only in these digesters with wheat straw biochar and sheep manure biochar and not in the controls (Figure 4) supports this hypothesis. It is unlikely that wheat straw biochar and sheep manure biochar are acting as inert materials in these digesters. Studies have shown the addition of inert, non-conductive sand particles does not increase butyrate concentrations or increase lag times (Cruz Viggi et al., 2017). Lower dosages of the two poor-performing biochars or their inclusion in low-solids digesters would be expected to lower the concentration of the inhibiting inorganic elements. This may explain the improved performance of low-solids digesters amended with biochar produced from dairy manure, chicken litter and vermicompost (Jang et al., 2017; Pan et al., 2019; Wang et al., 2017).

The physical properties of biochar may also have detrimental affects on the digester performance. The addition of wheat straw biochar, with its low bulk density (65 ± 2 kg/m^3, Table 1) increases the working volume of the bulk sludge by approximately 130%. By comparison, the higher bulk densities of wood pellet and sheep manure biochar result in only a 13% increase in volume. Even if inhibition due to the elemental composition of wheat straw biochar can be alleviated, digesters with wheat straw biochar are expected to have poor performances on a volumetric efficiency basis.

3.6. Effect of biochar pre-loading

This study showed that the addition of biochar pre-loaded with microorganisms in a high-solids digester for 90 days (WP90H) is more effective than adding raw biochar into a digester. Improved methane yields occur with WP90H despite the bulk sludge having an order of magnitude higher concentrations

of methanogens and other microorganisms.

The presence of WP90H may have increased methane yields through the degradation of propionate and isovalerate degradation. The degradation of these VFAs is only thermodynamically feasible if the concentration of the products formed during their oxidisation, formate and hydrogen, are kept low by the scavenging activity of partnering methanogens (Cruz Viggi et al., 2014; de Bok et al., 2004). Microorganisms in close proximity, as observed in SEM imagery, increases the likelihood of these syntrophic partnerships occurring (de Bok et al., 2004). In addition, WP90H may be facilitating propionate degradation via DIET between propionate/isovalerate-oxidising bacteria and *Methanosaetaceae*. Electrons donated from the oxidation of these VFAs may be transferred to and used by *Methanosaetaceae* for the reduction of bicarbonate into methane (Rotaru et al., 2014). It is likely a combination of partnering microorganisms in close proximity and the presence of DIET is allowing for increased propionate degradation.

The accumulation of propionate is often associated with a high organic load (initial VS per kilogram, or litre bulk sludge) and is common during the startup of digesters (Griffin et al., 1998). As high-solids digesters are generally operated in batch mode (Batstone and Jensen, 2011; Riggio et al., 2017), a start-up process must occur for every new batch (30–50 days), therefore, the degradation of propionate for additional methane production is crucial for the operation of these systems.

The method used for pre-loading has substantial effects on the anaerobic digester performance. The addition of WP30L had a much smaller effect compared with WP90H. This may be explained by the time used for pre-loading. WP90H had an additional 60 days for pre-loading and six times higher concentration of microorganisms attached to its surface (Figure 6). Therefore, the likelihood that both propionate/isovalerate oxidising bacteria and methanogens were attached on the same piece of biochar or within proximity would be higher. This may explain why WP30L did not decrease propionate or isovalerate concentrations. An additional consideration expected to facilitate microbial attachment is the total ammonia-nitrogen concentration in the pre-loading digester. Higher total ammonia-nitrogen concentrations occurs in high-solids digesters. The higher concentrations induces environmental stress

which would promote the attachment of microorganisms onto a solid surface and the formation of biofilms where microorganisms are in close proximity and protected from environmental stress (Petrova and Sauer, 2012). Therefore a combination of longer digestion time and higher total ammonia-nitrogen concentration likely resulted in additional microbial attachment on WP90H compared with WP30L.

4. Conclusions

The addition of biochar produced from wood pellets improves performance of high-solids anaerobic digesters. Biochar produced from wheat straw and sheep manure may introduce inorganic elements into the bulk sludge, due to their high ash content, which can inhibit methane generation. Wood pellet biochar pre-loaded with microorganisms for 90 days in a high-solids digester further improves methane yields compared with raw wood pellet biochar. This likely occurs through increased degradation of propionate and isovalerate. The time and digester environment used for microbial pre-loading effects the concentration of microorganisms on pre-loaded biochar which impacts its beneficial properties.

5. Supplementary Data

E-supplementary data of this work can be found in online version of the paper.

6. Acknowledgements

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Table 1: Properties of biochar produced from three parent materials.

Parameter	Wood pellet		Wheat straw		Sheep manure	
	mean	SD	mean	SD	mean	SD
Total solids (wt %)	96	1.0	90	0.5	94	0.3
Volatiles (% of TS)	4.6	0.7	12.6	2	7.9	0.3
Ash (% of TS)	0.3	0.4	14	2.0	58	0.2
pH	9.4	0.2	10.2	0.03	11.0	0.10
Total alkalinity (g-$CaCO_{3eq}$/kg)	7.3	2.1	16	1.7	28	6.4
Bulk density (kg/m^3)	329	3	65	2	225	4

Table 2: Elemental composition of biochar from three parent materials.

Parameter	Wood pellet	Wheat straw	Sheep manure
Carbon (wt% of TS)	89	63	34
Hydrogen (wt% of TS)	1.9	2	1
Nitrogen (wt% of TS)	0.2	1	2
Oxygen (wt% of TS)	8.7	20	5.5
H/C (mol/mol)	0.26	0.40	0.34
O/C (mol/mol)	0.07	0.24	0.12
Ca (g/kg-TS)	3.6	4	44
K (g/kg-TS)	0.7	39	43
Mg (g/kg-TS)	0.9	3	11
Na (g/kg-TS)	0.6	2	15
S (g/kg-TS)	0.1	2	4
Al (g/kg-TS)	0.2	1	5.9
B (g/kg-TS)	0.2	0	0.1
Cu (g/kg-TS)	0.8	0	0.1
Fe (g/kg-TS)	0.3	3	5.0
Mn (g/kg-TS)	0.1	0	0.5
P (g/kg-TS)	0.1	5	17.5
Zn (g/kg-TS)	0.0	0	0.4

Concentrations of As, Cd, Co, Cr, Mo, Ni, Pb and Se were below the detection limit of 0.01 g/kg-TS.

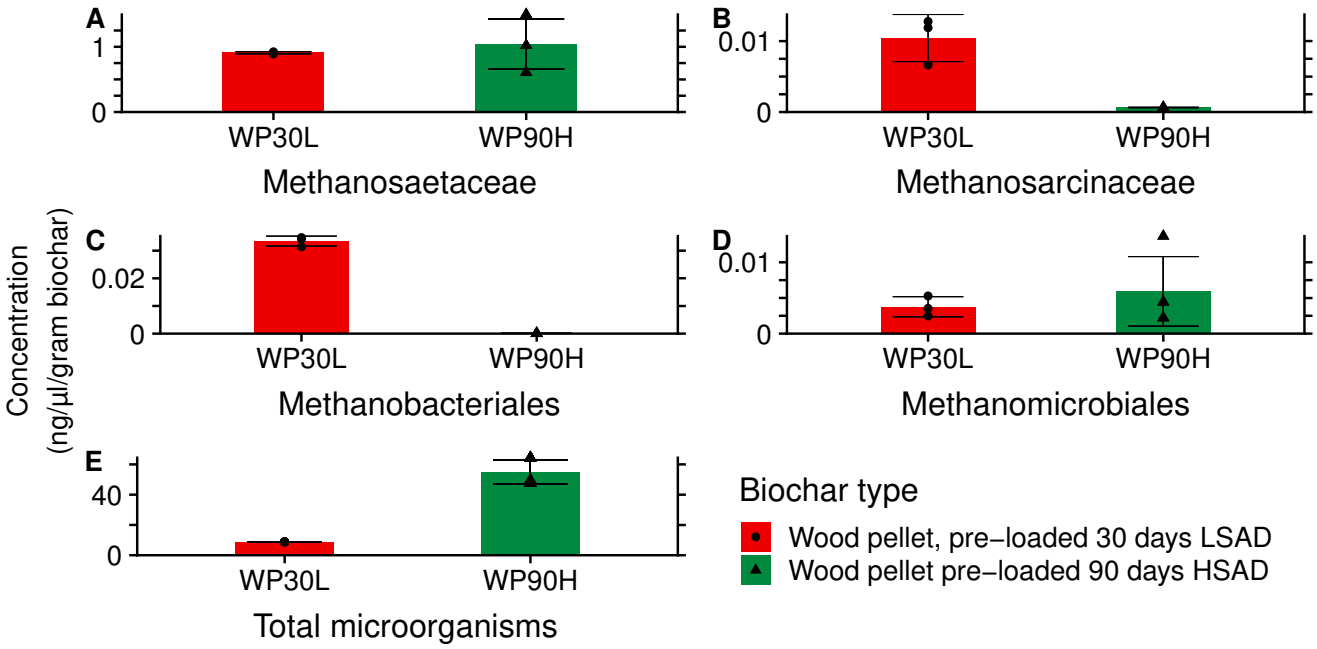

Figure 1: Concentration of methane-generating microorganisms (A-D) and total microorganisms (E) attached to two types of pre-loaded wood-pellet biochar prior to their addition in digesters for the methane production assay. The columns show the mean value, the error bars show the standard deviation and markers show the range from biological replicates.

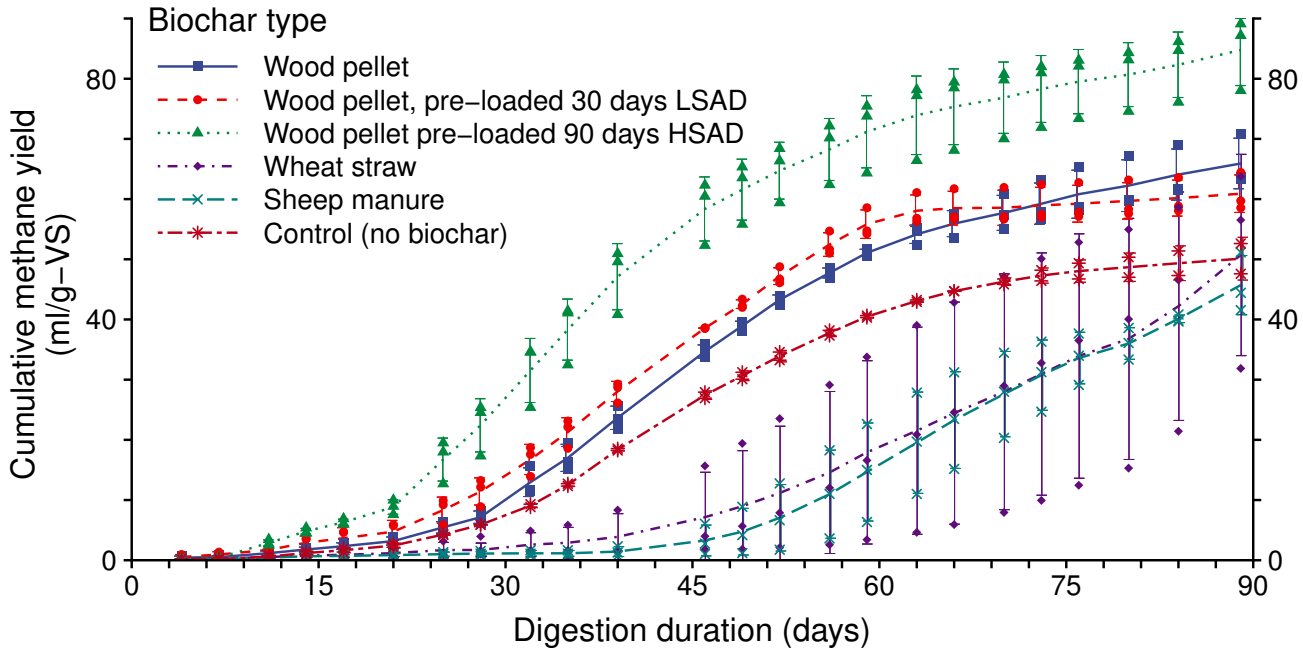

Figure 2: The cumulative methane yield over 90 days, normalised based on initial the volatile solids (VS) content of poultry litter and inoculant, from high-solids digesters with biochar and the control digesters (without biochar). The lines show the mean, error bars show the standard deviation and markers show the range from biological replicates.

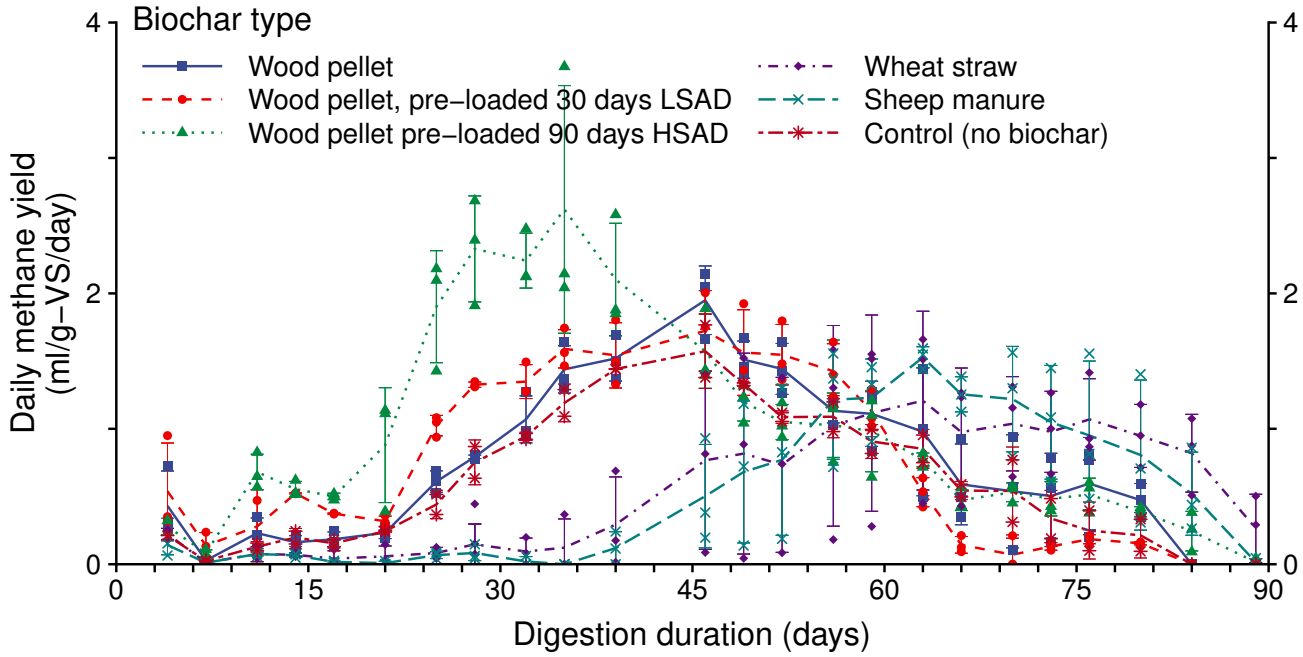

Figure 3: The daily methane yield over 90 days, normalised based on initial the volatile solids (VS) content of poultry litter and inoculant, from high-solids digesters with biochar and the control digesters (without biochar). The lines show the mean, error bars show the standard deviation and markers show the range from biological replicates.

Table 3: Summary of the Gompertz model parameters for digesters with varying biochar types, wood pellet (WP), wheat straw (WS), sheep manure (SM), wood pellet biochar pre-loaded for 30 days in a low-solids digester (WP30L) and 90 days in a high-solids digester (WP90H).

Biochar type	Lag time (days)		Maximum daily production rate ($ml\text{-}CH_4/g\text{-}VS/day$)		Potential yield ($ml\text{-}CH_4/g\text{-}VS$)	
	mean	SD	mean	SD	mean	SD
WP	24.0	0.5	1.6	0.04	67.8	1.0
WP30L	21.9	0.6	1.7	0.1	63.2	0.8
WP90H	16.8	0.8	2.1	0.1	84.2	1.4
WS	39.5*	2.5	1.2	0.1	91.1	25.6
SM	46.7	1.3	1.2	0.1	58.4	7.6
Control (no biochar)	25.1	1.5	1.4	0.1	59.2	2.4

* Estimated from two biological replicates as one replicate did not fit to the Gompertz model.

Table 4: Chemical conditions of the bulk sludge after 90 days in digesters with varying biochar types, wood pellet (WP), wheat straw (WS), sheep manure (SM), wood pellet biochar pre-loaded for 30 days in a low-solids digester (WP30L) and 90 days in a high-solids digester (WP90H)

Biochar type	pH		Total ammonia-nitrogen (g/kg)		Free ammonia-nitrogen (g/kg)		Volatile fatty acids (g/kg)		Total alkalinity (g-$CaCO_{3eq}$/kg)	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
WP	8.9	0.04	5.3	1.2	2.7	0.5	15.1	4.7	14.7	1.3
WP30L	8.8	0.04	6.5	0.4	2.9	0.3	27.1	2.1	14.9	1.2
WP90H	8.9	0.05	5.6	1.2	2.9	0.6	6.9	0.7	15.9	0.4
WS	8.9	0.02	4.6	0.8	2.3	0.4	23.2	2.7	11.3	3.0
SM	8.9	0.06	4.9	0.5	2.4	0.2	21.8	4.6	12.3	1.2
Control (no biochar)	8.9	0.00	5.1	1.3	2.6	0.6	20.3	2.2	12.8	1.1

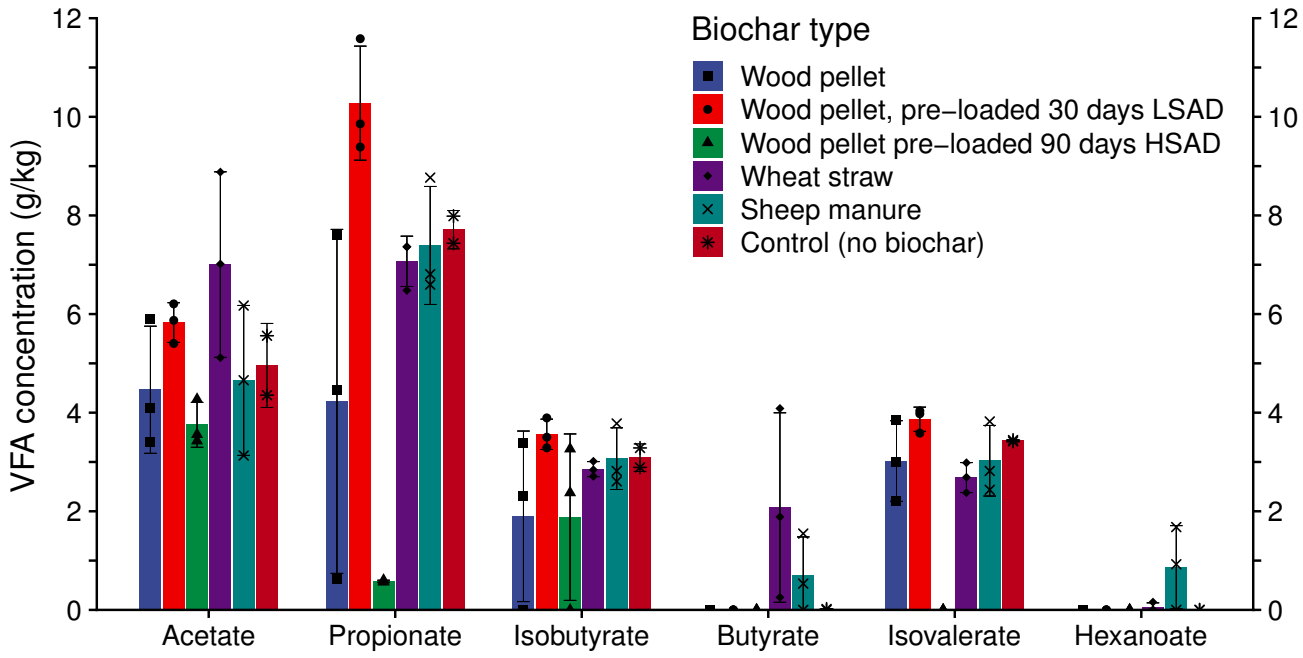

Figure 4: The volatile fatty acid (VFA) concentration in the bulk sludge after 90 days. The columns show the mean, error bars show the standard deviation and the markers show the range of values from the biological replicates.

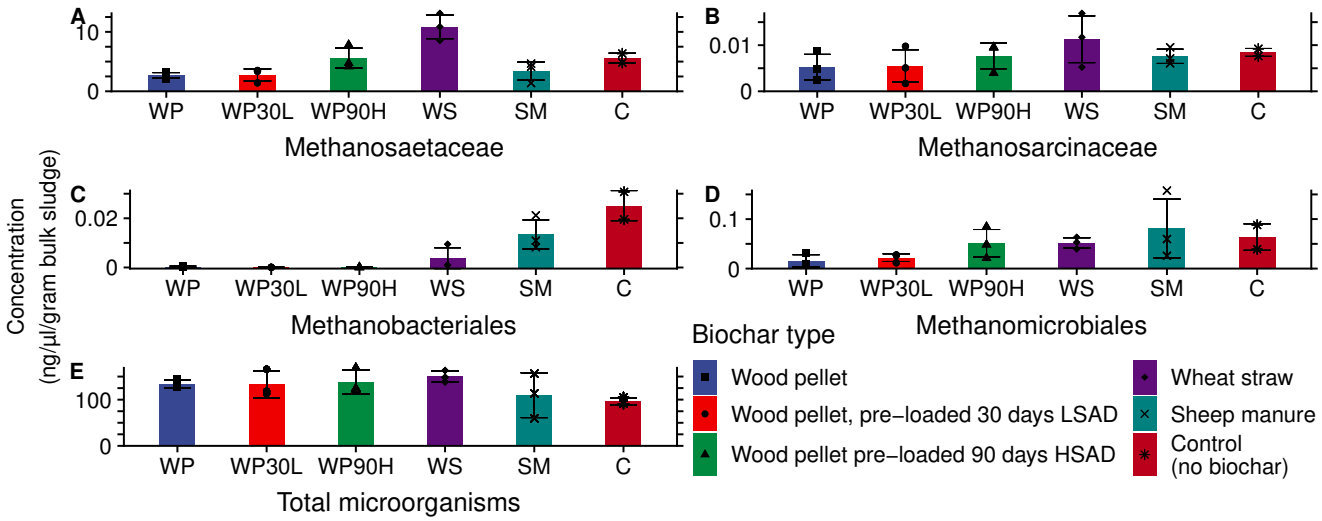

Figure 5: The concentration of methane-generating microorganisms (A-D) and total microorganisms (E) in the bulk sludge. The columns show the mean, the error bars show the standard deviation and markers show the range from biological replicates.

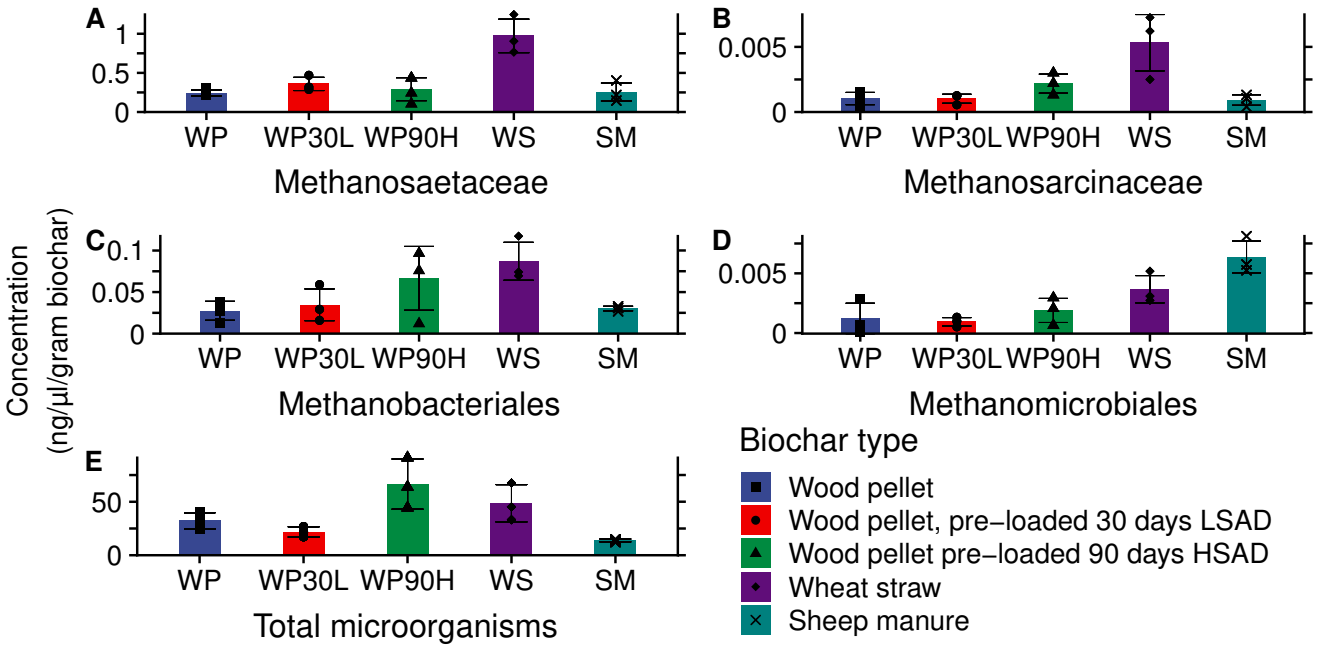

Figure 6: The concentration of methane-generating microorganisms (A-D) and total microorganisms (E) attached to the biochar. The columns show the mean, the error bars show the standard deviation and markers the show range from biological replicates.