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DOI: https://doi.org/10.1016/j.wasman.2018.02.022

Kirby, M.E, Theodorou, M.K., Brizuela, C.M., Huntington, J.A., Powles, J. and Wilkinson, R.G. 2018. The anaerobic digestion of pig carcase with or without sugar beet pulp, as a novel on-farm disposal method. *Waste Management*.

17 February 2018

The anaerobic digestion of pig carcase with or without sugar beet pulp, as a novel on-farm disposal method

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Abbreviations

Anaerobic digestion (AD), animal by-products (ABP), bovine spongiform encephalopathy (BSE), colony-forming units (CFU), carbon:nitrogen ratio (C:N), European Commission (EC), European Food Safety Authority (EFSA), feedstock (FS), fresh weight (FW), higher organic loading rate (-H), lower organic loading rate (-L), mixed feedstock (M), organic loading rate (OLR), pig carcase material (PCM), sugar beet pulp (SBP), total solids (TS), United Kingdom (UK), volatile fatty acids (VFA), volatile solids (VS).

Abstract

Anaerobic digestion was investigated as a potential method for on-farm disposal of fallen stock (pig carcases), degrading the carcase material to produce biogas and digestate. The effects of feedstock (sugar beet pulp or pig carcase material or a 50:50 mix) and organic loading rate (50g-TS L^{-1} or 100g-TS L^{-1}), during mesophilic (35^{o}C)

anaerobic digestion were investigated. Anaerobic digestion was achieved for all experimental treatments, however the pig carcase material at the higher organic loading rate produced the second highest methane yield (0.56Nm^3 kg-VS^{-1} versus a range of 0.14-0.58Nm^3 kg-VS^{-1} for other treatments), with the highest percentage of methane in total biogas (61.6% versus a range of 36.1-55.2% for all other treatments). Satisfactory pathogen reduction is a legislative requirement for disposal of carcase material. Pathogens were quantified throughout the anaerobic digestion process. *Enterococcus faecalis* concentrations decreased to negligible levels (2.8 $\log_{10}$ CFU g-TS^{-1}), whilst *Clostridium perfringens* levels remained unaffected by treatment throughout the digestion process (5.3±0.2 $\log_{10}$ CFU g-TS^{-1}).

Keywords – pig carcase; anaerobic digestion; animal by-product; carcase disposal; fallen stock.

1.0 Introduction

In 2016, there were approximately 147 million pigs in Europe (Eurostat, 2017), with 4.4 million pigs (DEFRA, 2016) farmed in the United Kingdom (UK). Fallen stock are defined as livestock which have died of natural causes or euthanised on-farm, and are therefore not fit for human consumption. The average mortality rates on UK pig farms are 5.4% sows, 12.2% pre-weanlings, 2.8% during rearing and 2.7% in the finishing herd (AHDB Pork, 2016). This results in a substantial quantity of fallen stock annually which requires safe, legal disposal.

Traditionally, European livestock farmers disposed of fallen stock and animal by-products (ABP) by on-farm open-burning and/or burial (Bansback, 2006). In 1984, bovine spongiform encephalopathy (BSE) appeared in UK cattle (Bansback, 2006) and in 1991, it was established that prions (the BSE causing agent) could remain infective within the soil for up to 3 years following carcase burial (Brown and Gajdusek, 1991). Subsequently, the ingestion of BSE-infected material was linked to the development of variant Creutzfeldt-Jakob disease in humans (Fox and Peterson, 2004). In order to reduce the risk of transmission within the cattle population and to humans, in 2002 the Commission Regulation (European Commission (EC)) No. 1774/2002 prohibited on-farm burning and burial for all fallen stock, irrespective of species susceptibility to prion diseases. This legislation required farmers to use alternative methods of disposal; either on-farm incineration, off-site incineration or off-site rendering. These methods (a) increased the cost of fallen stock disposal to farmers, (b) raised concerns regarding their negative environmental impact and (c) reduced farm biosecurity due to frequent movement of potentially contaminated vehicles between farms and fallen stock collection centres (Massé *et al.,* 2008).

Kirby *et al*. (2010) surveyed UK livestock (dairy, beef, sheep and pig) farmers to assess their compliance with EU fallen stock regulations and concluded that illegal disposal occurred for 13.7% for fallen stock, 19.5% for aborted foetuses/stillborns and 57.6% for placentas. The European Food Safety Authority (EFSA) provides scope for consideration and approval of new/novel methods for carcase disposal and storage. For a method to be considered, it must provide scientific evidence to demonstrate a

71 sufficient reduction in disease risk (of 5.0 $\log_{10}$ orders of magnitude), in specific key
72 animal and human health pathogens (EFSA, 2010).
73
74 Anaerobic digestion (AD) is commonly used to treat animal slurries, human sewage
75 (wastewater), municipal wastes and food wastes (Alvarez and Lidén, 2008). The AD
76 pasteurisation process can also destroy some pathogens (Escudero *et al.,* 2014) and
77 subsequently if compliant with legislation, the pasteurised digestate can be applied to
78 agricultural land as a fertiliser/soil conditioner (Salminen and Rintala, 2002). In relation
79 to protein-rich feedstocks, a number of investigators have examined the feasibility of
80 using AD for the treatment of slaughterhouse wastes (Jensen *et al*., 2014 and Ortner *et*
81 *al*., 2015), specified risk material (potentially prion-infected spinal cord material)
82 (Gilroyed *et al.,* 2010) and rendered ABP (Bayr *et al.,* 2012). Carcase material and
83 slaughterhouse wastes are ideal substrates for AD due to the high contents of organic
84 matter, protein and lipids (Palatsi *et al.,* 2011, Bayr *et al.,* 2012). However, the digestion
85 of high protein content feedstocks can produce high ammonia and volatile fatty acids
86 (VFA) concentrations, which can inhibit biogas production (Sung and Lui, 2003 and
87 Nielsen *et al.,* 2007). Moreover, carcase material differs from these previously
88 examined feedstocks, as complete carcases also contains bones, teeth and intestinal
89 content. Few authors have considered the possibility of using the AD process on-farm to
90 digest whole animal carcases. Massé *et al*. (2014) used AD at psychrophilic
91 temperatures (20 and 25°C) to successfully digested whole porcine carcases. Yuan *et al*.
92 (2012) digested carcase fractions, mixed with macerated carcase trimmings (without
93 intestinal contents) at mesophilic temperatures and demonstrated limited methane
94 yields.

Anaerobic digestion is currently not an EFSA approved carcase disposal method as the AD process conditions are unlikely to destroy prions. However, pigs and poultry are not susceptible to prion-infection via natural, oral infection routes (Ryder *et al.,* 2000), although experimental transmission can occur using artificial transmission routes (intracranial, intravenous and intraperitoneal) (Groschup *et al.,* 2007). Therefore, the AD process may be a suitable method for on-farm disposal of pig and poultry carcases. There is a research gap associated with the effective digestion of porcine carcases at mesophilic temperatures, particularly where co-digestion with carbohydrate-rich feedstocks are used to improve process stability and biogas yields. The objective of this research was to investigate the potential of AD for the disposal of pig carcase material (PCM), with and without sugar beet pulp (SBP) as an additional, highly-digestible carbon source. To establish if the AD process would be a suitable novel method for fallen stock on-farm, the research also investigated the potential for destruction of key indicator pathogens (*Enterococcus faecalis*, *Clostridium perfringens* and *Salmonella* spp), i.e., those described as pathogens in Commission Regulation (EC) No. 1774/2002 and Commission regulation (EC) No. 142/2011.

2.0 Materials and Methods

2.1 Reactor design

Six, cylindrical stainless steel bench-top AD reactors (458mm height, 210mm diameter), each with a working volume of 10L and head-space capacity of 2.6L (total volume 12.6L) were used for digestion studies. A schematic diagram of the exterior surface and interior paddle stirrer configuration of a reactor is shown in Figure 1a and

119 the reactors are shown photographically in Figure 1b. The reactors had 3 wall ports
120 spaced evenly down the cylindrical wall to allow digestate sampling from different
121 levels (top, middle and bottom). The head plate contained a feed port, sampling ports, a
122 gas nipple and a gas-tight paddle stirrer. The gas nipple permitted biogas from the
123 reaction vessel to be collected via tubing into a 5L capacity gas-tight Teflon bag
124 (35x26.5cm) that was both sealable and detachable such that it could be removed for
125 gas analyses. When biogas production was excessive, more than 1 bag could be
126 connected to each reactor. Reactors were intermittently mixed (for fifteen minutes in
127 every hour, except during feeding) using a paddle stirrer connected to a direct current
128 motor (TGE 511, Denso, The Netherlands) that enabled complete vortex mixing at 30
129 rotations per minute. Reactors were heated using thermostatic regulators connected to
130 an externally insulated electric heating jacket which, except for the head plate and base,
131 completely covered the reaction vessel (Figure 1b).
132
133 **2.2 Experimental design**
134 The experiment was a 3x2 factorial block design repeated over 3 separate periods each
135 of 53 days duration. The 6 reactors were cleaned and reassembled between periods. The
136 effects of 6 individual treatments, comprising 3 feedstocks and 2 organic loading rates
137 (OLR), were investigated at mesophilic temperature (34±2°C). Each treatment occurred
138 once in each period. The feedstocks were either SBP, PCM or a mixed (M) feedstock
139 containing 50:50 SBP:PCM on a w/w total solids (TS) basis. Reactors were fed on
140 alternate days during the feeding phase, with an OLR of either 50g-TS L^{-1} (low, -L) or
141 100g-TS L^{-1} (high, -H).
142

143 Each 53-day period consisted of 3 discrete phases. The first phase was an
144 acclimatisation phase lasting for 3 days. At the start of this phase pre-heated reactors
145 were filled with 5L of fresh, non-pasteurised digestate taken from a typical commercial
146 mesophilic AD food waste plant. The purpose of the acclimatisation phase was to
147 ensure initial biogas production from the introduced digestate. The second phase, the
148 feeding phase, ran for 20 days post-acclimatisation. During the feeding phase, reactors
149 were fed once every 2 days receiving a total of 10 feeds. Each feed consisted of 25 or
150 50g-TS^{-1} feedstock in 500ml with distilled water. Therefore, a total of 250 or 500g-TS^{-1}
151 feedstock (in 10x500ml aliquots) were fed to each reactor. The third phase of the
152 experiment was the non-feeding phase which ran for 30 days post-feeding. This non-
153 feeding phase was included to ensure EFSA requirements were met with regard to
154 carcase degradation and pathogen destruction, i.e., prior to emptying, the AD system
155 would need to be sealed for a specified period after the last carcase had been added.
156

157 **2.3 Preparation of feedstocks**

158 A whole gilt (approximately 50kg) was obtained from a commercial pig unit at the
159 University of Nottingham, slaughtered, quartered and frozen (-20^{o}C). The entire,
160 quartered carcase (including all organs and digesta contents) was macerated twice
161 (13mm then 4mm mincing plates) using a Wolfking type C-160 Universal grinder
162 (Boyd International Limited, Buckie, UK) and thoroughly mixed. Commercial SBP
163 animal feed was milled through a 2mm diameter dry mesh screen (Christy & Norris 8"
164 Laboratory & Soil Mill, Ipswich, UK). For the purpose of description in this
165 manuscript, a feedstock is defined as the material that is fed to the reactor, whereas a
166 treatment refers to the combination of a feedstock and its organic loading rate.

167

168 **2.4 Experimental routine (per period)**

169 The reactors were assembled and nitrogen was flushed through the feed port for 1
170 minute to remove oxygen (in air). Non-pasteurised AD digestate (6 x 5.5L aliquots) was
171 collected on the first day of the acclimatisation phase and a 500ml subsample was
172 removed from each aliquot for subsequent analyses. The remaining 5L of digestate was
173 transferred immediately into 1 of the pre-warmed reactors; this process was repeated 5
174 more times to fill all reactors.

175

176 Following the acclimation phase, digestate samples were taken on day 1 of the feeding
177 phase (prior to the addition of feedstock) through the feed port on the head plate
178 (500ml). Subsequently feeding commenced (500ml aliquots) via the feed port and
179 continued every other day for a total of 10 feeds. Biogas volume and composition were
180 measured daily. Reactor temperature and pH were measured on the first day of the
181 feeding phase and subsequently on alternate days, via the feed port; these measurements
182 were made prior to addition of feedstock. At 10 day intervals (days 11, 21, 31, 41 and
183 51) a further 200ml sample was removed from the lower wall port of the reactor and
184 analysed for pH, ammonium and VFA. Additional samples (50ml) were taken from the
185 same lower wall port at the start and end of the feeding phase and at the end of the non-
186 feeding phase and analysed for TS, volatile solids (VS) and enumeration of
187 *Enterococcus faecalis, Clostridium perfringens* and *Salmonella* spp. To calculate TS (g)
188 and VS (g) content of the digestate, reactors were weighed before the experiment
189 commenced (empty weight), at the end of the feeding phase and at the end of the
190 experiment.

The three pathogens used in this experiment were chosen to comply with the requirements of Commission Regulation (EC) No. 1774/2002, being infective to both humans and animals. Commission regulation (EC) No. 142/2011 states that the feedstock is pasteurised prior to or the digestate is pasteurised following the AD process (70°C for 1 hour). However, Commission Regulation (EC) No. 1774/2002 states that liquid material containing fallen stock and/or ABP should be sterilised (133°C, 300kPa pressure for 20 minutes). To determine if pasteurisation or sterilisation would achieve pathogen destruction, additional samples were taken for microbial counts at the end of the non-feeding phase and either pasteurised or sterilised.

2.5 Chemical analyses

The volume of biogas collected in Teflon bags was measured using a dry test gas meter (Shinagawa Corporation, Tokyo, Japan) and all gas volume data was normalised to 20°C and 1 atmosphere. Methane composition was measured using a PGD3-IR infrared portable gas analyser (Status Scientific Controls, Mansfield, England).

Digestate pH was measured using a pH probe (Jenway, UK) in conjunction with a temperature probe; the reactor temperature was measured with a thermometer. Both pH and temperature measurements were taken via the feed port in the head plate. The ammonium concentration of the liquid digestate was measured using the Watson and Galliher, (2001) methodology for Kjeldahl on a Foss Kjeltec 1035 sampler (FOSS, Hillerød, Denmark). The VFA concentrations were determined using the method described by Cruwys *et al*. (2002) on a Perkin Elmer Clarus 500 gas chromatograph

fitted with a Nukol free fatty acid phase fused-silica capillary column (30m x 0.25mm ID, film thickness 0.25µm, category number 24107, Supelco Ltd, Dorset, UK) (Cruwys *et al.,* 2002).

The TS (g) content of the feedstocks and digestate samples were determined using freeze-drying (to constant weight), with the VS and carbon contents measured as a percentage of the TS. The VS content was calculated as the weight of TS (g) lost following ashing (at 550ºC) of the freeze dried sample overnight in a muffle furnace (Carbolite, Hope Valley, England). The total carbon content was analysed using a sulphur and carbon analyser, Leco SC-144 DR (LECO, Stockport, England) with samples (0.05g) weighed into a crucible boat of known weight and combusted at 1000ºC. Total nitrogen (for carbon:nitrogen (C:N) ratios) was analysed using the Dumas method (Watson and Galliher, 2001) on a nitrogen/protein Leco FP-528 (LECO, Stockport, England). Crude protein was determined by multiplying the nitrogen content (percentage of TS) by 6.25 (Jones, 1931). Ether extract content was measured by the standard method for crude fat (Horwitz, 2000) using a Soxtec HT 1043 extraction unit (FOSS, Hillerød, Denmark). All analysed values were adjusted for the daily calibration against the appropriate calibration curve.

2.6 Microbial analyses

Analyses for pathogenic microorganisms were conducted at the Eclipse Scientific Group laboratories (Telford, England) using the culture methods of detection for *E. faecalis* (BS 4285 3.11.1985) (BSI, 1985), *C. perfringens* (BS EN ISO 7937:2004) (BSI, 2004) and *Salmonella* spp. (BS EN ISO 6579:2002) (BSI, 2002). Bacterial

numbers were enumerated as the number of colony-forming units (CFU) g-fresh weight (FW^{-1}) and converted to CFU g-TS^{-1} for each treatment. The data was transformed to log_{10} CFU g-TS^{-1}.

2.7 Statistical analyses

Data were analysed by ANOVA as a 3x2 factorial block design, main effects being feedstock and OLR, with blocking through replicate (3 replicates per treatment). Statistical analyses were conducted using GenStat version 17 with a significance level of $P \leq 0.05$, using Fisher's predicted least significant difference. Factorial ANOVA was used to compare cumulative, biogas analyses data. For pH, ammonium, VFA and pathogen concentrations it was possible to analyse the data by ANOVA in two distinctly different ways: either to investigate the treatment effects at each of the three time points (days 1, 21 and 51) both with and without covariate adjustment or by using repeated measures analysis to investigate the behaviour of treatment effects over the time course of the study. Both approaches were employed, but the repeated measures analysis was considered the more meaningful, and is presented as the analysis of choice. The rationale for this was because the antedependence ANOVA revealed that individual day differences were influenced by the previous sampling date, rather than the effect of treatment itself on each sampling date. This rendered analysis of data from individual dates less appropriate.

3.0 Results and Discussion

3.1 Treatment Chemical Composition

262 The chemical composition of each feedstock and the feedstock formulation per
263 treatment are presented in Table 1. The crude protein contents for SBP and PCM were
264 reasonably similar to previously published values of 103g-TS L^{-1} for SBP (Ziemiński *et*
265 *al.,* 2012) and 375g-kg^{-1} for PCM (Whittemore and Kyriazakis, 2006).
266
267 **3.2 Influence of treatment on reactor pH and ammonium concentrations**
268 Table 2 records pH and ammonium concentrations over the time course of the
269 experiment. Related factors (time, feedstock, OLR) did not interact significantly to
270 influence the change in pH that was observed during the time course of the experiment
271 (Table 2). However, the pattern of pH change over time was statistically the same for
272 each of the six treatments tested (Table 2, P=0.002). In each case, the pH declined from
273 the start of the experiment (mean 8.09) until the end of the feeding phase (mean 7.38),
274 before significantly increasing by the end of the experiment (mean 7.83). The pH values
275 at the end of the experiment were statistically the same as those on day 1 (Table 2).
276 There was also a significant main effect of feedstock across all time periods whereby
277 the non-porcine containing feedstock elicited a significantly (P=0.024) lower pH than
278 the two porcine containing feedstocks (Table 2). Declines in pH in AD reactors are
279 usually associated with increased acidity caused by the production of VFA by
280 acidogenic microorganisms in poorly buffered digestate (Wang *et al.,* 2009). This was
281 particularly noticeable for the non-porcine containing treatments (SBP-H and SBP-L)
282 due to a combination of high carbohydrate and/or reduced protein contents, reducing the
283 buffering capacity compared to porcine containing treatments.
284

285 For ammonium concentrations, related factors (time, feedstock, OLR) interacted
286 significantly ($P<0.001$) to influence the change in concentrations observed during the
287 time course of the experiment. Ammonium concentrations were statistically the same at
288 the start of the experiment (mean 3.02 g L^{-1}). Thereafter, the pattern of change over time
289 and across all treatments was influenced to a large extent by the presence or absence of
290 porcine material in the treatment. During the feeding phase, ammonium concentrations
291 increased significantly in reactors fed porcine material (PCM-H, PCM-L, M-H),
292 whereas they declined significantly in the reactor receiving the lowest concentration of
293 porcine material (M-L) and the two SBP treatments (Table 2). Differences between the
294 extent of change in porcine containing treatments were also significant (PCM-H >
295 PCM-L = M-H > M-L). Corresponding ammonium concentrations in reactors fed
296 porcine containing material did not alter significantly during the non-feeding phase of
297 the experiment (Table 2). In the two reactors fed non-porcine containing treatments
298 (SBP-H, SBP-L), ammonium concentrations decreased significantly during the feeding
299 phase before increasing significantly by the end of the experiment (Table 2).
300 Ammonium concentrations in reactors fed these two treatments, at both day 21 and day
301 51 of the experiment, were significantly lower than the concentrations accumulating in
302 reactors fed all other treatments (Table 2). There was also a significant main effect of
303 feedstock across all time periods which showed that the two porcine containing
304 feedstocks accumulated significantly higher ammonium concentrations than the SBP
305 feedstock ($P=0.001$) according to the relationship (PCM = M > SBP) (Table 2). As was
306 to be expected, there was an exceptionally good linear relationship between feedstock
307 protein content and reactor ammonium concentrations (Figure 2). The linear
308 relationship demonstrates that carcase protein hydrolysis, particularly at the higher

309 OLR, was not limited by other physiological factors or treatment characteristics. This
310 effect was also noted by Resch *et al.* (2011) when digesting ABP.
311
312 Protein is most effectively degraded to ammonium at neutral pH (Shu-guang *et al.,*
313 2007), with digestion stability dependent upon the C:N ratio of the feedstock and its
314 subsequent buffering capacity. Chen *et al*. (2008) described an optimum C:N ratio of
315 20:1. The C:N ratios for feedstocks used in the current work ranged from SBP 25:1, M
316 10:1 and PCM 7:1 (Table 1). High protein feedstocks can enhance reactor buffering
317 capacity, as production of ammonium from protein degradation increases pH (Escudero
318 *et al*., 2014). Sugar beet pulp had a low nitrogen content (Table 1) so could not buffer
319 against the pH decreased caused by the accumulating VFA concentrations. In keeping
320 with these results, Shu-guang *et al.* (2007) working on the dry mesophilic AD of high
321 protein (25%) and carbohydrate (28%) dog food also noted a similar pH decrease (due
322 to the high rate of VFA production) and subsequent increase in pH. Ammonium levels
323 for all six treatments were comparable to the combined ammonium and soluble
324 ammonia concentrations of 1.7-5.7 g L^{-1} published by Edström *et al*. (2003) for the
325 digestion of animal slurries and slaughterhouse wastes, with SBP feedstock containing a
326 lower protein content displaying the lowest yields of Edström *et al*. (2003).
327
328 **3.3 Influence of treatment on reactor volatile fatty acid concentration**
329 Throughout the experiment, acetic acid was the most abundant VFA for all treatments,
330 followed by substantially lower concentrations of propionic and remaining acids
331 (butyric and valeric) (Table 3). In general, the pattern of VFA change was similar over
332 time and across all treatments. Lower levels of VFA (0.4-1.0 g L^{-1} acetic acid; 0.05-0.11

333 g L^{-1} propionic acid; 0.12-0.24 g L^{-1} remaining acids) at the start of the experiment
334 accumulated by day 21 to higher levels (4.7-9.3 g L^{-1} acetic acid; 0.42-2.95 g L^{-1}
335 propionic acid; 0.77-2.71 g L^{-1} remaining acids). Thereafter, VFA concentrations
336 declined and by the end of the experiment levels were similar to those recorded at day 1
337 (Table 3). There was one notable exception to the general response. In reactors fed with
338 the SBP-H treatment, VFA concentrations increased both during the feeding phase and
339 non-feeding phase of the experiment (Table 3). With the exclusion of the SBP-H
340 treatment, the range of VFA concentrations at day 51 were not dissimilar to those
341 recorded at the start of the experiment (0.1-0.4 g L^{-1} acetic acid; 0.04-0.13 g L^{-1}
342 propionic acid; 0.11-0.39 g L^{-1} remaining acids). Statistical analysis revealed that
343 related factors (time, feedstock, OLR) interacted significantly ($P<0.001$) to influence the
344 change in VFA concentrations during the time course of the experiment. The pattern of
345 VFA change across all treatment showed significant increases ($P<0.001$) for most VFA
346 concentrations to the end of the feeding phase. However, there were three exceptions;
347 butyric and valeric acid concentrations (i.e., the remaining acids in Table 3) from
348 reactors fed SBP-L, PCM-L and M-L treatments, while increased, were not significantly
349 different to day 1 values (Table 3). Thereafter, and in all but one treatment (SBP-H),
350 VFA concentrations declined significantly or, for the three exceptions mentioned above,
351 remained unaltered; at the end of the experiment they were not significantly different
352 the values recorded on day 1.
353
354 **3.4 Influence of treatment on reactor biogas production**
355 The biogas accumulation profiles for all treatments during the time course of the
356 experiment are shown in Figure 3. All treatments resulted in biogas production. Biogas

accumulated linearly during the feeding phase of the experiment, except for SBP-H where the gas profile began to plateau before the end of the feeding phase. Thereafter, the rate of gas accumulation slowed and tended towards a plateau (Figure 3). Table 4 records biogas and methane yields and the percentage of methane in biogas at the end-points (51 days) of the gas accumulation profiles. Feedstock and OLR interacted significantly to influence the total biogas yield for each treatment (Table 4). Unlike the pH and VFA interactions, it was not possible to distinguish absolutely between porcine and non-porcine containing treatments on the basis of their total biogas yields. The two feedstocks containing just porcine material produced the significantly ($P<0.001$) highest total biogas yields (PCM-L>PCM-H). Treatments M-H, M-L and SBP-L produced significantly lower but similar total biogas yields (M-H= M-L= SBP-L) and the SBP-H treatment produced the lowest total biogas yield (Table 4).

Methane yields from each treatment were also influenced by feedstock and OLR but to a lesser extent than total biogas yields (Table 4). It was therefore possible to segregate treatments on the basis of porcine containing feedstocks. These four treatments resulted in the production of significantly higher methane values than the two treatments that contained just SBP (Table 4). For porcine containing treatments, the two treatments containing just porcine material (PCM-L and PCM-H) produced similar but significantly ($P=0.031$) higher methane yields than the two mixed treatments (M-L and M-H), which were similar to each other but significantly lower than the porcine only treatments. Moreover, the two treatments which contained the higher OLR in the porcine grouping (PCM-H and M-H) were also similar to each other (Table 4).

381 The percentage of methane in biogas was calculated for each of the three replicates for
382 each treatment. It was possible to distinguish between porcine containing and non-
383 porcine containing treatments on the basis of the percentage of methane in biogas. The
384 four porcine containing treatments resulted in the production of biogas with
385 significantly (P=0.018) greater methane percentage than the two SBP treatments, with
386 SBP-L having significantly greater percentage of methane in biogas than SBP-H (Table
387 4). The percentage of methane in the biogas for PCM-H (61.6%) was significantly
388 (P=0.018) greater than for the other three porcine containing treatments which were
389 similar to each other (range 53.7-55.2%; Table 4).
390
391 In general, while interactions were apparent, the results presented in Table 4 can be
392 summarised to show that feedstocks containing just SBP produced significantly lower
393 biogas and methane yields with significantly lower methane percentage in biogas than
394 the four porcine containing treatments. Where SBP was mixed with porcine material,
395 (M-H and M-L), this resulted in a reduction (often significant) in the yield of gases and
396 in the methane percentage in comparison to the treatments that contained just porcine
397 material (PCM-L and PCM-H). Thus, while PCM-H produced the greatest yield of
398 biogas, PCM-L produced significantly less biogas but with a significantly higher
399 methane percentage. These results can be contrasted to those obtained for acetic acid
400 production (Table 3) where five of the treatments resulted in levels of acetic acid (the
401 principal methanogenic pre-cursor) that were not different from each other but were
402 lower than the yield produced by the SBP-H. Nielsen *et al.* (2007) demonstrated a
403 similar increase in VFA concentrations, decrease in pH and reduction in methane

404 production due to the increased feeding rate of SBP, inhibiting methanogens and
405 causing an unstable digestion process (Wang *et al.*, 2009).
406
407 The percentage of methane in total biogas for the four porcine containing treatments
408 ranged from 53.7-61.6% (Table 4). These values were slightly lower than those
409 obtained for mesophilic digestion of slaughterhouse waste (66-69%) (Ortner *et al*.,
410 2015). However the methane yields were considerably higher for PCM containing
411 treatments (range 0.44–0.58Nm^3 kg-VS^{-1}) compared to previously published data of
412 0.36m^3 kg-VS^{-1} for cattle manure and potentially prion-infected spinal cord material at a
413 lower OLR of 30g-VS L^{-1} (Gilroyed *et al.,* 2010); the mono-digestion of pig blood of
414 0.44-0.48Nm^3 kg-VS^{-1}; pig intestinal contents of 0.45-0.66Nm^3 kg-VS^{-1} and grease
415 separation of 0.43-0.50Nm^3 kg-VS^{-1} (Ortner *et al*., 2015). Additionally, the methane
416 yield for PCM containing feedstock was higher than the excepted methane yields for
417 food waste 0.27m^3 kg-VS^{-1} and for garden waste 0.53m^3 kg-VS^{-1} (Browne *et al.,* 2014).
418
419 **3.5 Influence of the AD process on the persistence of *Clostridium perfringens,***
420 ***Enterococcus faecalis* and *Salmonella* spp.**
421 Although *Salmonella* spp. are found within carcases and are often associated with food
422 poisoning (Côté *et al*., 2006), they were not detected in any of the samples taken from
423 the bench-top AD reactors.
424
425 For *E. faecalis*, related factors (time, feedstock, OLR) did not interact significantly
426 ($P>0.050$) to influence the change in CFU counts observed during the time course of the
427 experiment (Table 5). *E. faecalis* was detected on days 1 and 21 of the experiment.

428 There was a marginal decrease in CFU counts across all treatments by day 21 but the
429 numbers detected at the end of the acclimatisation and feeding phases (day 1 and 21)
430 were not significantly different. By the end of the non-feeding phase (day 51) CFU
431 counts fell to negligible levels (<2.8 $\log_{10}$ CFU g-TS^{-1}; equivalent to <20 CFU g-FW^{-1})
432 for all treatments, demonstrating that sufficient time had elapsed under unfavourable
433 conditions and with no further addition of *E. faecalis* during the non-feeding phase to
434 destroy known concentrations (Table 5). Consequently, due to their negligible
435 concentration at day 51, it was not possible to quantify the effect of pasteurisation and
436 sterilisation on *E. faecalis* populations. EFSA (2010) requires a 5.0 $\log_{10}$ reduction in
437 specific pathogens for the pathogen destruction process to be deemed safe. However
438 due to insufficient natural occurrence of *E. faecalis* in digestate samples, while AD was
439 clearly effective, it was not possible to achieved the required reduction in population
440 numbers.
441
442 For *C. perfringens*, related factors (time, feedstock, OLR) did not interact significantly
443 ($P<0.001$) to influence the change in CFU counts observed during the time course of the
444 experiment (Table 5). *C. perfringens* were detected on days 1, 21, 51 of the experiment
445 and after the pasteurisation but not the sterilisation procedures. The CFU counts for *C.*
446 *perfringens* from all treatments remained unaltered throughout the experiment but a
447 significant decrease ($P<0.001$) of just 1 $\log_{10}$ unit in population numbers occurred
448 following pasteurisation of the digestate (Table 5). *Clostridium* can form spores which
449 are resistant to heat inactivation (Sahlström *et al.,* 2008). Pasteurisation was not
450 therefore effective at destroying *C. perfringens* to the 5.0 $\log_{10}$ reduction level required
451 by Commission regulation (EC) No. 142/2011. These results confirm the previous

findings of Sahlström *et al*. (2008) where sporulation of *C. perfringens* occurred following pasteurisation of digestate at 70°C for 1 hour. However, when day 51 samples were sterilised (133°C, 300kPa pressure for 20 minutes), *C. perfringens* detection levels were negligible (<2.5 $\log_{10}$ CFU g-TS^{-1}; equivalent to <10 CFU g-FW^{-1}) (Table 5). Sterilisation was therefore effective in meeting Commission regulation (EC) No. 142/2011. The average main effect across all time periods (including pasteurisation) showed a significantly higher (P=0.044) concentration of *C. perfringens* in the feedstock containing just SBP, compared to porcine containing feedstocks (Table 5). These significances in CFU counts were marginal, possibly suggesting that *C. perfringens* survived the pasteurisation process to a moderately greater extend in feedstocks that did not contain porcine material (Table 5).

Overall, the results presented suggest that the natural abundance of microorganisms in the initial digestate and their development over time was not sufficient to demonstrate the EFSA required 5.0 $\log_{10}$ reduction for the development of a novel storage method for fallen pigs (Commission regulation (EC) No. 142/2011). Similarly, Costa *et al*. (2017) also demonstrated a reduction in pathogen concentration within animal slurries and digestate during storage, but natural pathogen concentration was not great enough to demonstrate the required EFSA 5.0 $\log_{10}$ reduction.

3.6 Suitability of the AD process in relation to policy

Anaerobic digestion of PCM potentially provides a sustainable alternative method for the disposal of fallen pigs. This research has demonstrated that PCM can be digested anaerobically to produce methane, with and without an additional carbon source such as

476 SBP. Methane yields were significantly higher (P=0.031) for treatments PCM-L and
477 PCM-H (Table 4; mean 0.57Nm^3 kg-VS^{-1}) and according to the literature, were
478 substantially higher than mixed cattle/pig slaughterhouse waste (0.06m^3 kg-VS^{-1}) or
479 fruit and vegetable wastes (0.45m^3 kg-VS^{-1}) (Ortner *et al*., 2015). Digestion of SBP was
480 not ideal, as biogas yields were lowest for feedstock SBP and biogas yields for M-H
481 were reduced in comparison to PCM-L, which contained the same quantity of carcase
482 material (Table 4). Therefore any future research digesting carcase material would not
483 benefit from co-digestion with SBP.
484
485 In relation to pathogen destruction, *E. faecalis* was destroyed to negligible levels by the
486 end of the experiment, however *C. perfringens* concentrations remained at unacceptable
487 levels throughout the experiment. Commission Regulation (EC) No. 1774/2002 requires
488 a 5.0 $\log_{10}$ reduction of pathogens (Commission Regulation (EC) No. 1774/2002) for a
489 novel method to be approved. Therefore further research is needed using pathogen
490 spiked AD reactors to known pathogen concentrations to enable the observation of >5.0
491 $\log_{10}$ reduction in pathogen numbers. Additionally pre- and post-treatment processes
492 could be investigated (see Commission regulation (EC) No. 142/2011) to help achieve
493 the required 5.0 $\log_{10}$ reductions for key pathogens.
494
495 Potential benefits of using AD for the disposal of pig carcases include the fact that the
496 digester would need to be emptied less frequently compared to the current, frequent
497 collection (almost daily) methods for fresh/stored carcases. Reducing collection
498 frequency would improve the farm's biosecurity (Massé *et al.,* 2008), as there would be
499 fewer vehicle movements onto the farm which could spread disease. Additionally, the

biogas could be converted into electricity or heat to be used on-farm, further reducing farm costs. Based upon the methane yields from the PCM-H treatment, if the methane was combusted under 1 standard atmosphere at 25°C with 100% engine efficiency, it would produce 6.8kWh kg-VS^{-1}. Further research would be required to optimise the digestion process, using both pre- and post-treatment of PCM, to determine the effects this has upon the destruction of key pathogens required by EFSA. Pilot scale trials would then have to be conducted on-farm with the full operational and safety requirements completed.

4.0 Conclusion

This research demonstrates that PCM can be effectively digested at 35°C with and without a carbon source, to produce significant quantities of total biogas with methane concentrations ranging from 40.3-67.5%. The treatment PCM-H (100g-TS L^{-1}) was the most suitable treatment in relation to process stability and biogas yield, producing 0.85Nm^3 kg-VS^{-1} total biogas and 0.56Nm^3 kg-VS^{-1} methane (Table 4). Addition of carbon, in the form of SBP, was not necessary to successfully digest PCM at these OLR. Naturally occurring populations of *Salmonella* spp., *E. faecalis* and *C. perfringens* on these feedstocks were not sufficiently large to permit verification of EFSA requirements for alternative disposal methods. In the case of *C. perfringens*, due to the survival of heat-tolerant spores, it was evident that pasteurisation alone did not present a suitable method for pathogen destruction. Further research is therefore required to investigate pathogen destruction both pre- and post-AD processing of PCM, prior to pilot scale AD trials on porcine farms.

Acknowledgements

Financial support was provided by Agricultural and Horticulture Development Board, Pork Division (Kenilworth, UK) and Harper Adams University to fund this research. Special thanks are given to Dr Mewa S. Dhanoa for statistical advice and to Mr John Chapman, Miss Tracey Lewis and the laboratory staff at Harper Adams University for their help with the laboratory analyses. Kirby, Brizuela, Huntington, Powles and Wilkinson designed and carried out the experiment and analysed the results, whilst Kirby and Theodorou conducted the statistical analyses, drafted and revised the manuscript.

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655

656 Table and Figure Captions

657 Table 1 Chemical composition of the feedstocks; sugar beet pulp and pig carcase
658 material, and feedstock formulations
659 Table 2 The effect of feedstock and organic loading rate on repeated measures of pH
660 and ammonium concentration (g L^{-1}) throughout the experiment

661 Table 3 The effect of feedstock and organic loading rate on repeated measures of net
662 volatile fatty acid concentrations (g L^{-1}) throughout the experiment
663 Table 4 The effect of feedstock and organic loading rate on cumulative total biogas and
664 methane yields (Nm^3 kg-VS^{-1}) and methane (%) of total biogas
665 Table 5 The effect of feedstock and organic loading rate on split-plot analysis of
666 variance of *Enterococcus faecalis* and repeated measures of *Clostridium perfringens*
667 concentrations (log_{10} CFU g-TS^{-1}) at the end of the acclimatisation, feeding and non-
668 feeding phases; for the non-feeding determinations, measurements were made on
669 samples pre- and post-pasteurisation and moist heat sterilisation
670 Figure 1a Schematic diagram of the exterior surface and internal paddle stirrer
671 configuration of the 12.6 litre anaerobic reactors
672 Figure 1b Photograph of the six operating reactors
673 Figure 2 Linear relationship between the total crude protein content fed to reactors (g)
674 and accumulated ammonium concentration (g L^{-1}) in reactors, at day 21 and 51, across
675 all treatments
676 Figure 3 The effect of feedstock and organic loading rate on total biogas yield (Nm^3 kg-
677 VS^{-1}) throughout the experiment. Values presented are cumulative, 5-day summations.
678 The SEM across all 5-day summations ranged from 0.01-0.10.

679 Table 1

Chemical compositions (g kg^{-1})	SBP	PCM
Total solids	866.7	337.2
Volatile solids	766.4	315.1
Total carbon	400.8	569.6
Total nitrogen	16.0	76.9
Crude protein	100.0	480.6
Ether extract	6.6	403.2

Feedstock formulations (g)	SBP-L	SBP-H	PCM-L	PCM-H	M-L	M-H
Fresh weight	291	582	736	1472	513	1026
Total solids	252	504	248	496	250	500
Volatile solids	228	456	227	454	228	456
Volume of water (ml)	4709	4418	4264	3529	4487	3973
Total carbon	101	202	141	282	121	242
Total nitrogen	4	8	19	38	12	24
Ether extract	2	4	97	194	49	98
Carbon:nitrogen ratio	25:1	25:1	7:1	7:1	10:1	10:1

680 Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (organic loading
681 rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}.
682
683

684

685

Table 2

	Treatments						P values			
pH	SBP-L	SBP-H	PCM-L	PCM-H	M-L	M-H	Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	8.07^{b}	8.09^{b}	8.07^{b}	8.10^{b}	8.10^{b}	8.10^{b}				
Day 21	7.44^{a}	6.38^{a}	7.79^{a}	7.63^{a}	7.44^{a}	7.60^{a}	0.002	0.077	0.248	0.196
Day 51	7.90^{b}	6.48^{b}	8.18^{b}	8.23^{b}	8.06^{b}	8.15^{b}				
Average main effect across all time periods							FS		OLR	FS x OLR
	7.80^{A}	6.98^{A}	8.02^{B}	7.99^{B}	7.87^{B}	7.95^{B}	0.024		0.144	0.088
Ammonium (g L^{-1})							Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	3.05^{d}	3.01^{d}	3.01^{d}	3.05^{d}	2.98^{d}	3.04^{d}				
Day 21	1.75^{a}	1.76^{a}	3.32^{e}	5.23^{f}	2.53^{c}	3.30^{e}	0.002	<0.001	<0.001	<0.001
Day 51	2.03^{b}	2.03^{b}	3.50^{e}	5.35^{f}	2.73^{c}	3.53^{e}				
Average main effect across all time periods							FS		OLR	FS x OLR
	2.28^{A}	2.27^{A}	3.28^{C}	4.54^{D}	2.75^{B}	3.29^{C}	<0.001		<0.001	<0.001

Repeated measurement analysis (split-plot-in time) over all time points. Numerator and denominator degrees of freedom were scaled by the Greenhouse-Geisser epsilon before calculating F-ratio probability. FS, Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}. Day 1 (end of the acclimatisation phase), day 21 (end of the feeding phase) and day 51 (end of the non-feeding phase). Mean data interactions in columns and rows (lower case superscripts) and average treatment data in rows (upper case superscripts) with the same superscript are not significantly different (P>0.050).

Table 3

Acetic acid ($g\ L^{-1}$)	Treatments						P value			
	SBP-L	SBP-H	PCM-L	PCM-H	M-L	M-H	Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	0.8^{a}	0.8^{a}	0.8^{a}	0.5^{a}	1.0^{a}	0.4^{a}				
Day 21	4.7^{b}	9.3^{c}	5.6^{b}	5.9^{b}	4.7^{b}	5.1^{b}	<0.001	0.001	0.002	0.002
Day 51	0.2^{a}	11.5^{d}	0.1^{a}	0.4^{a}	0.1^{a}	0.1^{a}				
Average main effect across all time periods							FS		OLR	FS x OLR
	1.9^{A}	7.2^{B}	2.2^{A}	2.3^{A}	1.9^{A}	1.9^{A}	<0.001		<0.001	<0.001
Propionic acid ($g\ L^{-1}$)							Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	0.11^{a}	0.06^{a}	0.05^{a}	0.07^{a}	0.05^{a}	0.05^{a}				
Day 21	0.42^{b}	2.95^{d}	0.42^{b}	2.51^{c}	0.59^{b}	2.30^{c}	<0.001	<0.001	<0.001	<0.001
Day 51	0.05^{a}	3.73^{e}	0.05^{a}	0.13^{a}	0.05^{a}	0.04^{a}				
Average main effect across all time periods							FS		OLR	FS x OLR
	0.19^{A}	2.24^{C}	0.17^{A}	0.91^{B}	0.23^{A}	0.80^{B}	<0.001		<0.001	<0.001
Remaining acids (butyric and valeric) ($g\ L^{-1}$)							Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	0.24^{a}	0.24^{a}	0.22^{a}	0.24^{a}	0.23^{a}	0.12^{a}				
Day 21	0.77^{a}	1.77^{b}	0.79^{a}	2.71^{c}	0.80^{a}	1.67^{b}	<0.001	<0.001	<0.001	<0.001
Day 51	0.11^{a}	3.89^{d}	0.11^{a}	0.39^{a}	0.11^{a}	0.11^{a}				
Average main effect across all time periods							FS		OLR	FS x OLR
	0.37^{A}	1.97^{C}	0.37^{A}	1.11^{B}	0.38^{A}	0.63^{AB}	0.022		<0.001	0.022

Repeated measurement analysis (split-plot-in time) over all time points. Numerator and denominator degrees of freedom were scaled by the Greenhouse-Geisser epsilon before calculating F-ratio probability. FS, Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}. Day 1 (end of the acclimatisation phase), day 21 (end of the feeding phase) and day 51 (end of the non-feeding phase). Mean data interactions in columns and rows (lower case superscripts) and average treatment data in rows (upper case superscripts) with the same superscript are not significantly different (P>0.050).

Table 4

	Treatments						P value		
Biogas produced (Nm^3 kg-VS^{-1})	SBP-L	SBP-H	PCM-L	PCM-H	M-L	M-H	FS	OLR	FS x OLR
Biogas yield	0.72^{b}	0.33^{a}	0.97^{d}	0.85^{c}	0.74^{b}	0.77^{b}	<0.001	<0.001	<0.001
Methane yield	0.33^{b}	0.14^{a}	0.58^{e}	0.56^{de}	0.44^{c}	0.46^{cd}	<0.001	0.039	0.031
Methane (%) in biogas	42.5^{b}	36.1^{a}	53.7^{c}	61.6^{d}	54.1^{c}	55.2^{c}	<0.001	0.540	0.018

FS, Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}. Mean data in rows with the same superscript are not significantly different (P>0.050).

Table 5

Enterococcus faecalis (log_{10} CFU g-TS^{-1})	Treatments						P value			
	SBP-L	SBP-H	PCM-L	PCM-H	M-L	M-H	Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	6.1	6.1	6.4	6.1	6.1	6.2	0.701	0.317	0.967	0.579
Day 21	6.0	5.8	5.4	5.6	5.5	5.2				
Day 51	N	N	N	N	N	N	-	-	-	-
Pasteurised day 51	N	N	N	N	N	N	-	-	-	-
Sterilised day 51	N	N	N	N	N	N	-	-	-	-
Average main effect across all time periods							FS		OLR	FS x OLR
	6.1	5.9	5.9	5.8	5.8	5.7	0.492		0.481	0.989
Clostridium perfringens (log_{10} CFU g-TS^{-1})							Time	Time x FS	Time x OLR	Time x FS x OLR
Day 1	5.8^{b}	5.9^{b}	5.9^{b}	5.7^{b}	5.4^{b}	5.7^{b}				
Day 21	5.8^{b}	6.0^{b}	5.5^{b}	5.4^{b}	5.9^{b}	5.6^{b}	<0.001	0.769	0.792	0.949
Day 51	5.3^{b}	5.5^{b}	5.3^{b}	5.4^{b}	5.4^{b}	5.1^{b}				
Pasteurised day 51	5.1^{a}	4.6^{a}	4.3^{a}	4.0^{a}	4.3^{a}	4.1^{a}				
Sterilised day 51	N	N	N	N	N	N	-	-	-	-
Average main effect across all time periods, including pasteurised day 51							FS		OLR	FS x OLR
	5.5^{B}	5.5^{B}	5.2^{A}	5.1^{A}	5.3^{A}	5.1^{A}	0.044		0.420	0.812

Repeated measurement analysis (split-plot-in time) for *Clostridium perfringens* over all time points. Numerator and denominator degrees of freedom are scaled by the Greenhouse-Geisser epsilon before calculating F-ratio probability. FS, Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}. Day 1 (end of the acclimatisation phase), day 21 (end of the feeding phase) and day 51 (end of the non-feeding phase). Mean data interactions in columns and rows (lower case superscripts) and average treatment data in rows (upper case superscripts) with the same superscript are not significantly different (P>0.050). Pasteurised samples were held at 70°C for 1 hour and sterilised samples were held at 133°C for 20 minutes at 300kPa pressure. Colony-forming unit (CFU) counts that were negligible (N) equated to <20 CFU g-FW^{-1} (<2.8 log_{10} CFU g-TS^{-1}) for *Enterococcus faecalis* and <10 CFU g-FW^{-1} (<2.5 log_{10} CFU g-TS^{-1}) for *Clostridium perfringens.*

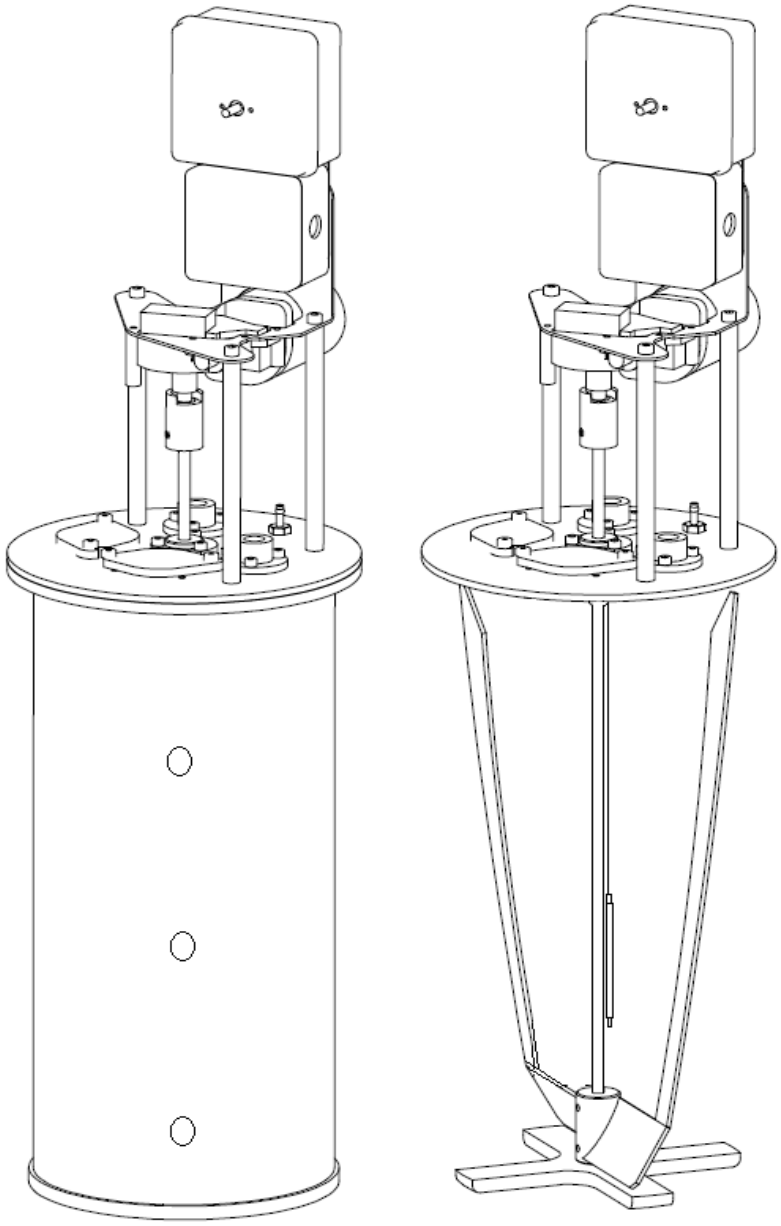

Figure 1a

Figure 1b

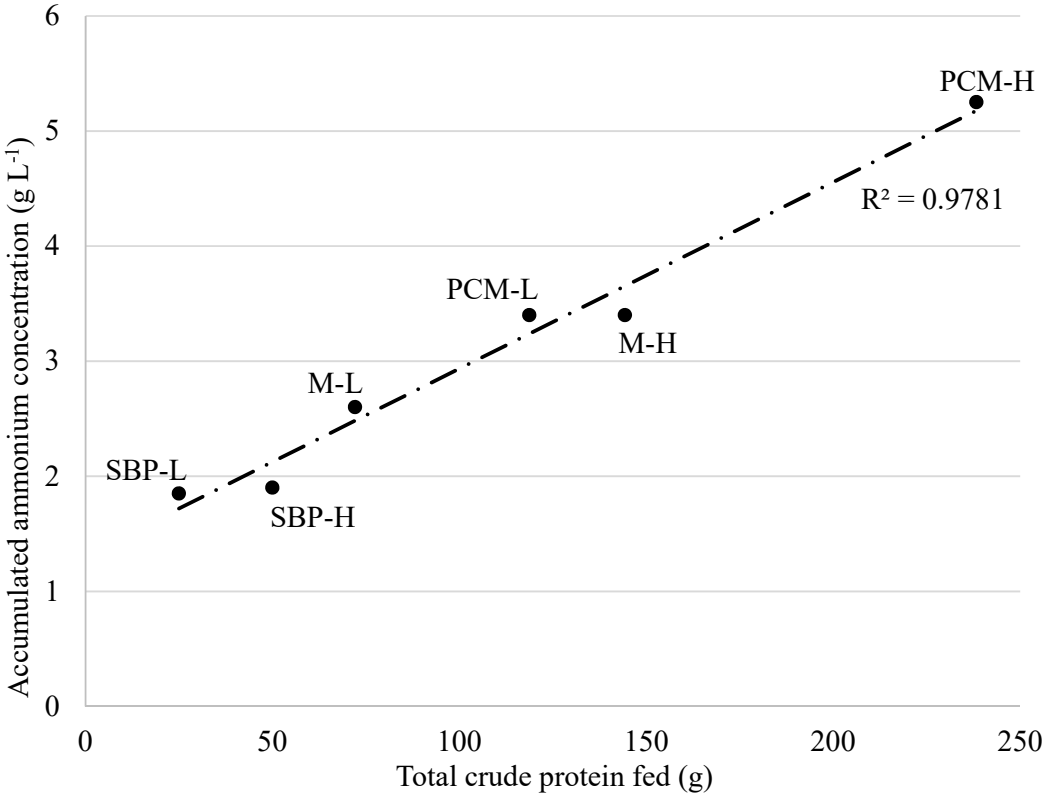

Figure 2

Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}.

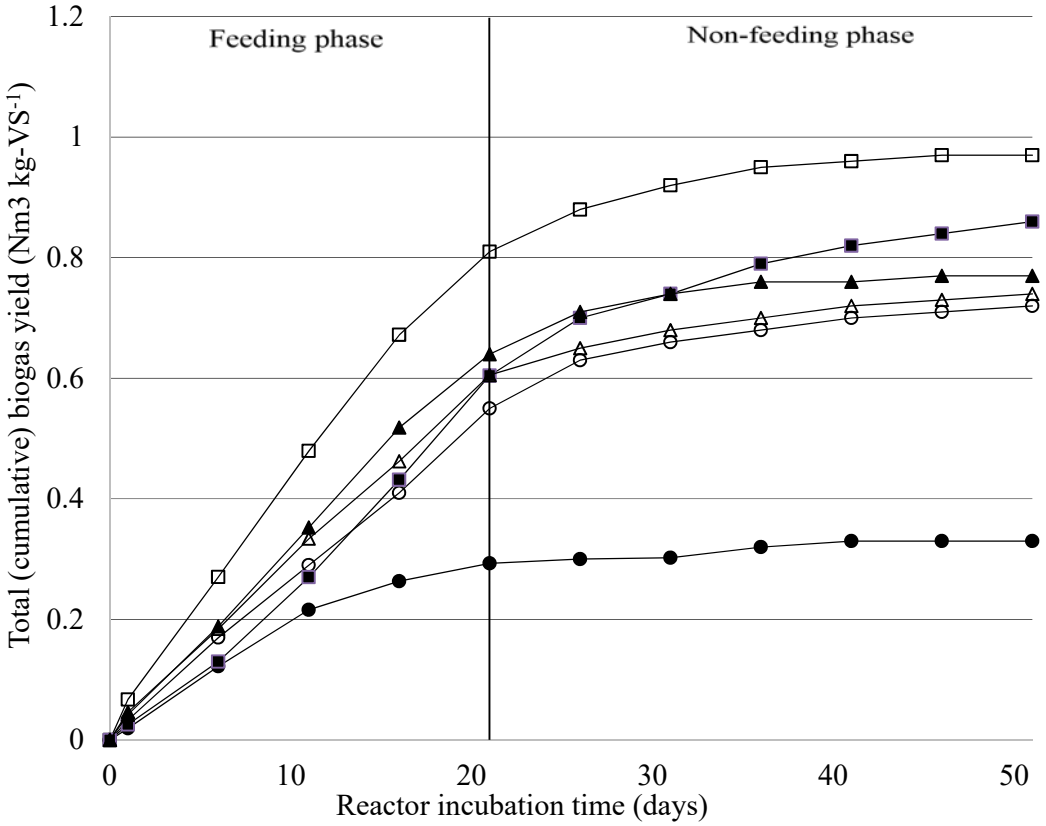

Figure 3
Feedstocks: SBP, sugar beet pulp; PCM, pig carcase material; M, mixed (50%:50% SBP: PCM). Feedstock formulations (OLR, organic loading rate): -L, Low 50 g-TS L^{-1}; -H, High 100 g-TS L^{-1}. Symbols: ○ SBP-L, ● SBP-H, □ PCM-L, ■ PCM-H, Δ M-L and ▲ M-HL.