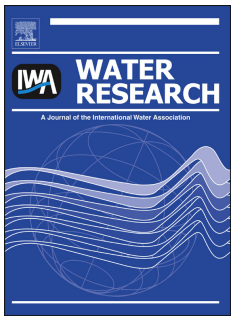
Skillman, L.C., Bajsa, O., Ho, L., Santhanam, B., Kumar, M. and Ho, G. (2009) ***Influence of high gas production during thermophilic anaerobic digestion in pilot-scale and lab-scale reactors on survival of the thermotolerant pathogens Clostridium perfringens and Campylobacter jejuni in piggery wastewater.*** **Water Research, 43 (13). pp. 3281-3291.**

Accepted Manuscript



Title: Influence of high gas production during thermophilic anaerobic digestion in pilot-scale and lab-scale reactors on survival of the thermotolerant pathogens Clostridium perfringens and Campylobacter jejuni in piggery wastewater.

Authors: L.C. Skillman, O. Bajsa, L. Ho, B. Santhanam, M. Kumar, G. Ho

PII: S0043-1354(09)00281-4

DOI: 10.1016/j.watres.2009.04.031

Reference: WR 7406

To appear in: *Water Research*

Received Date: 26 November 2008

Revised Date: 9 March 2009

Accepted Date: 20 April 2009

Please cite this article as: Skillman, L.C., Bajsa, O., Ho L., Santhanam B. , Kumar, M., Ho, G. Influence of high gas production during thermophilic anaerobic digestion in pilot-scale and lab-scale reactors on survival of the thermotolerant pathogens Clostridium perfringens and Campylobacter jejuni in piggery wastewater., Water Research (2009), doi: 10.1016/j.watres.2009.04.031

Influence of high gas production during thermophilic anaerobic digestion in pilot-scale and lab-scale reactors on survival of the thermotolerant pathogens *Clostridium perfringens* and *Campylobacter jejuni* in piggery wastewater.

L.C. Skillman[a*], O. Bajsa[b], L. Ho[b], B.Santhanam[c], M. Kumar[c], G. Ho[b]

[a]*Western Australian State Agricultural Biotechnology Centre (SABC), Murdoch University, South Street, Murdoch, Western Australia*

[b]*School of Environmental Science, Murdoch University, South Street, Murdoch, Western Australia*

[c]*South Australian Research and Development Institute (SARDI), Breakwell Laboratory, Roseworthy Campus, 5371, South Australia*

Abstract

Safe reuse of animal wastes to capture energy and nutrients, through anaerobic digestion processes, is becoming an increasingly desirable solution to environmental pollution. Pathogen decay is the most important safety consideration and is in general, improved at elevated temperatures and longer hydraulic residence times. During routine sampling to assess pathogen decay in thermophilic digestion, an inversely proportional relationship between levels of *Clostridium perfringens* and gas production was observed. Further samples were collected from pilot-scale, bench-scale thermophilic reactors and batch scale vials to assess whether gas production (predominantly methane) could be a useful indicator of decay of the thermotolerant pathogens *Clostridium perfringens* and *Campylobacter jejuni*. Pathogen levels did appear to be lower where gas production and levels of methanogens were higher. This was evident at each operating temperature (50, 57, 65 ^{o}C) in the pilot-scale thermophilic digesters, although higher temperatures also reduced the numbers of pathogens detected. When methane production was higher, either when feed rate was increased, or pH was lowered from 8.2 (piggery wastewater) to 6.5, lower numbers of pathogens were detected. Although a number of related factors are known to influence the amount and rate of methane production, it may be a useful indicator of the removal of the pathogens *Clostridium perfringens* and *Campylobacter jejuni*.

Keywords: anaerobic digestion; *Campylobacter jejuni; Clostridium perfringens*; piggery effluent; pathogen; thermophilic.

1. Introduction

Anaerobic digestion is widely used to treat wastewater and organic sludges as it reduces oxygen demand, generating both methane for fuel and rich organic manure. To make reuse efforts safe, it is necessary to remove bacterial pathogens present in the waste to acceptable levels. Thermophilic anaerobic digestion is often preferred as it offers a shorter residence time than ambient temperature (mesophilic) systems.

Several systems have been investigated to digest swine wastewater, some by co-digestion with other waste streams (reviewed by Sakar et al., 2009). These systems include: continuous stirred tank reactors (CSTR) similar to the benchscale reactors described in this study (Hansen et al., 1998; Kaparaju & Rintala, 2005); upflow anaerobic digesters (UASB) (Sanchez et al., 2005) and hydrid UASB (Lo et al., 1994); baffled anaerobic reactors (Boopathy, 1998); anaerobic sequencing batch reactor (ASBR) (Zhang et al., 1997); and two stage anaerobic digesters similar to the pilot-scale system described in this study (Zhang et al., 2000). The reactors above were operated between 25 and 60o^{o}C at volumes between < 1 L and 380 L, with an organic loading rate (OLR) of 0.9 to 15 g VS L^{-1} day^{-1} and hydraulic residence times of <1 to >100 days. Chemical oxygen demand (COD) removal ranged from 57 to 78% and volatile solid (VS) removal from 35 to 61%. Methane yields were reported between 22 and 360 ml CH_4 g^{-1} VS added (Sakar et al., 2009). Many studies on piggery wastewater highlight the inhibitory effects of high levels (1700 to 2300 mg L^{-1}) of ammonia-N on reactor performance (Hansen et al., 1998) but Zhang et al. (1997) reported no adverse effect on reactor performance at levels up to 2470 mg L^{-1} in an

ASBR. Although the factors which influence solubilisation during thermophilic anaerobic digestion have been well studied (Angelidaki et al., 1999; Batstone et al., 2002), studies on pathogen reduction in the treatment process have been given only secondary consideration (Vanotti et al., 2005).

Pathogen die-off in anaerobic treatment is influenced by many variables including sensitivity of the specific types of pathogens, temperature, retention time (reviewed by Bendixen, 1994), pH, free ammonia (Nielsen and Ahring, 2007), volatile fatty acid (VFA) concentration (Kunte et al., 1988; Salsali et al., 2008; Wilson et al, 2008), moisture content, mixing, pathogen density (Horan et al., 2004), availability of nutrients and the presence of other microorganisms (Sidhu et al., 2001; Wrigley 2004). In general, increasing digestion temperature reduces pathogen survival in an exponential pattern with concomitant die-off at longer hydraulic residence times. The extent of microbial inactivation also depends on the microorganisms, their age, media, pH and nutrient availability (Smith et al., 2005).

In some cases, increasing thermophilic digestion temperature is not feasible due to the cost of additional energy requirements. In other cases, increasing digestion temperature may be ineffective (Sahlstrom, 2003) or even undesirable against bacterial pathogens which have heat survival mechanisms. Although vegetative cells of *Clostridium perfringens* are susceptible to temperatures above 50 ^{o}C, their ability to form spores which can survive over an hour at 100 ^{o}C, allows them to resist thermophilic digestion (Olsen and Larsen, 1987; Burtscher et al., 1998). Some non-spore

100 formers have alternate mechanisms to resist heat, which in the case of
101 *Campylobacter jejuni*, involve the expression of heat shock proteins and
102 chaperonins (Stinzi 2003; Murphy et al., 2005). Both pathogens are known
103 to be present at high levels in piggery wastewater(Chinivisagum et al.,
104 2004). Preliminary studies in our laboratory suggested these two pathogens
105 in particular, survived thermophilic digestion at levels above their infective
106 dose and were selected to monitor die-off during thermophilic digestion. The
107 aim of this study was to investigate solubilisation of piggery wastewater and
108 maximise pathogen destruction. Factors affecting solubilisation and gas
109 production also appeared to influence pathogen destruction.

2. Materials and methods

2.1. *Outline, operation and sampling of pilot-scale thermophilic reactors*

The pilot-scale anaerobic digester system makes use of a two-stage (thermophilic then mesophilic) anaerobic digestion process. The first thermophilic digestion stage utilises two 130 L continuous flow stirred tank (CFST) reactors running in parallel. The mixing pump operates for 15 mins every hour to prevent solid build up and give thorough mixing of the digester contents. These CFST reactors are fed once-daily with 20 L raw piggery wastewater (0.5 to 3 % total dry solids w/v). They are insulated 175 litre 16Cr-13Ni-3Mo 3 mm thick stainless steel vessels, equipped with heating tape, thermocouples and a pressure relief safety valve. They operate a fixed 7 d hydraulic retention time (HRT), at temperatures between 55 and 70 ^{o}C.

Effluent is collected from the on-site holding sump prior to pumping to an anaerobic lagoon from a 500 head pig farm (0-2 year olds), which are housed in roofed pens and fed commercial pig feed. Sufficient effluent for each trial was collected from the sump and stored at 4 ^{o}C. Solids analyses and pH measurements were performed according to standard methods (American Public Health Association, 1999). Gas production was measured using positive displacement type gas meters with on-line logging of gas flow. The primary component of this device was a U-tube containing silicone oil (Dow Corning Pty. Ltd.), a three-way solenoid valve, float switch and a timer. The biogas produced accumulated at the top of one side of the U-tube and displaced the liquid so that the liquid level on

the other side increased. When the liquid on the other side reached a certain level, a float above the liquid activated a switch. This triggered three events simultaneously; 1) a signal sent to the counter to record the number of clicks and displayed it; 2) accumulated biogas was exhausted to atmosphere through the solenoid valve, which reset the liquid level, and 3) a timer was activated to allow biogas to escape and to allow liquid to reach a steady level. The timer was manually set at 3 seconds after which, the solenoid valve returned to its original position. During the vent cycle, the three-way solenoid valve isolated the reactor from the meter. Gas production rate was estimated by the volume of gas required to initiate one click multiplied by the number of clicks. Calibration was checked before and after each experimental run. All the tubes used in the experiment were Masterflex tygon tubes (No. 18) (Cole Parmer).

A Varian Star 3400 Model Gas Chromatograph equipped with a TCD was used for biogas analysis. High purity nitrogen gas was used as the carrier gas with detector and column temperature at 120 ^{o}C. Porapak Q 80/100 column of length 6 inch and tubing 1/8 inch SS were used. Gas chromatograms were recorded and processed. Retention times of hydrogen, carbon dioxide and methane were used for the detection of peaks. The operating conditions for the anaerobic system were chosen based on chemical oxygen demand (COD) reduction and nutrient removal capacity of the system (Lettinga and Hushoff Pol, 1991; Guerrero *et al*., 1999; Yu and Fang, 2001). Samples were collected for chemical analyses during process optimisation trials from the thermophilic reactors at different operating temperatures (55 and 70 ^{o}C). Determination of total (TS) and

volatile (VS) solids, total (TCOD) and soluble (SCOD) chemical oxidation demand, total kjeldahl nitrogen (TKN), total ammonia nitrogen (TAN), soluble (SP) and total phosphorus (TP) were carried out according to the standard methods (American Public Health Association, 1999). Volatile fatty acids (VFA) in the samples were analysed by a gas chromatograph with a flame ionisation detector and capillary column.

2.1.1. Sampling period 1

After allowing the system to operate through a minimum of four hydraulic retention time periods to achieve an hydraulic steady-state, 30 ml samples were collected from one thermophilic reactor, running between 56 and 58 ^{o}C at 7 d HRT. Samples included the raw effluent on day 1 and day 7 to check pathogen populations during effluent storage at 4 ^{o}C. The initial reactor sample was collected just after the effluent had been introduced into the thermophilic reactor and mixed thoroughly (T0). Remaining samples were collected at 24 hourly intervals for 7 further days (T1 to T7) and on day 12 (T8) but collected just before effluent addition (20 L) to the thermophilic reactor (130 L). All samples except T8 were frozen immediately after collection for real-time PCR quantitation and sample T8 was sent to Murdoch University on ice by overnight delivery for most probable number (MPN) enumeration.

2.1.2. Sampling period 2

As above, the system was operated for 4 HRT periods to achieve an hydraulic steady state. Thirty millilitre samples were collected from one thermophilic reactor, running at 65 ^{o}C, and 7 d HRT. Samples were collected at weekly intervals over 4 weeks before and after effluent addition

(20 L) to the thermophilic reactor (130 L). Samples included the raw effluent each week. All samples were frozen immediately after collection for real-time PCR quantitation.

2.1.3. Sampling period 3

As above, the system was operated for 4 HRT periods to achieve an hydraulic steady state. Thirty millilitre samples were collected from both thermophilic reactors on the same day, running at 50 ^{o}C and 7 d HRT over an 8-month period. Samples were collected immediately before effluent addition (20 L) to the thermophilic reactor (130 L). The samples were sent fresh to Murdoch University on ice by overnight delivery for MPN enumeration and quantitation by real-time PCR.

2.2. Outline, operation and sampling of lab-scale thermophilic reactors (CSTR)

Two continuous-flow, stirred thermophilic reactors (CSTR) were set-up using computer control (Labview, National Instruments) of the feed and effluent pumping as well as real-time data logging of temperature and gas production. Total volumes of 1.2 L were seeded with working volumes of 0.9 L of activated sludge, acclimatised to anaerobic conditions for 4 weeks, and fed with effluent, diluted with distilled water to a final COD feed concentration of 4.4, 7.2 and 12.9 g/L. The reactors were drained and fed intermittently (10 times in 48 h) at 2 d HRT. The lab-scale reactors were run at a short, 2d HRT, to encourage acidification, although this was achieved with synthetic wastewater in preliminary studies, acidification was not achieved with piggery wastewater due to its large buffering

capacity. The short HRT was used to investigate the degree of solubilisation and acidification, rather than maximise methane production. Four volumes of HRT turnover were allowed before active sampling of effluents and gas began for 8 consecutive days. The volume of biogas produced daily was measured as previously described. The biogas composition was analysed for hydrogen, methane and carbon dioxide using a Varian gas chromatograph as described above. The effluent samples were analysed for pH, alkalinity, total and soluble COD, total and volatile suspended solids, C2 to C6 volatile fatty acids, ammonia-nitrogen, soluble phosphorus and pathogen levels by real-time PCR. Student t-tests were used to statistically compare the reactors performance for solubilisation, acidification and methanogenesis.

2.3. Batch Vials

Piggery wastewater collected from the lab-scale thermophilic reactor at 10 d HRT was used to inoculate a mixed microbial community. To ensure there were adequate degradable volatile fatty acids as food source in the reactor effluent, 10 % v/v chilled raw piggery wastewater was added to the reactor effluent and divided into five 50 ml portions in 120 ml serum bottles capped with butyl rubber stoppers and crimped tightly with aluminium seals: control vial without pH adjustment; pH adjusted to 8.2 with concentrated hydrochloric acid; and pH 7.5; pH 7.0 and pH 6.5. The headspace was purged with nitrogen and degassed after equilibrating to 55 ^{o}C, before incubating in a shaking water-bath at 55 ^{o}C for 10 d. Gas volume was measured by plunger displacement and composition was analysed daily using a Varian gas chromatograph as above. From the gas

volume and methane concentration, cumulative methane production was calculated for each sample vial. At the conclusion of the experiment, samples were taken for pH, total and soluble COD, VFA and ammonia-nitrogen and real-time PCR analysis.

2.4. Organisms, growth conditions and most probable number (MPN) enumeration

Two isolates were used as positive controls in MPN estimations and to prepare enumerated cultures for real-time PCR standards. Firstly, *Campylobacter jejuni* subsp. *jejuni* strain NCTC 11351 was maintained at 4 ^{o}C on Skirrow agar (Skirrow, 1977) and subcultured every two weeks. Plates were grown for 48 h in sealed jars at 42 ^{o}C under microaerophilic conditions using a gas generating system (Oxoid, Hampshire, UK). For liquid culture and MPN enumeration, Preston broth was prepared as described previously by Bolton and Robertson (1982) according to the Australian Standards ^{TM} (AS 5013.6-2004), with the addition of FBP supplement (George et al., 1978). Duplicate dilution series were prepared for each sample in nutrient broth and added to test tubes containing Preston broth as per 3 test tube MPN methods described in the Australian standards ^{TM} (AS4276.1:2007). They were incubated for 4 h at 37 ^{o}C before the addition of selective antibiotic supplement (Amyl) (0.1 ml). The tubes were then incubated at 42 ^{o}C for a further 44 h. An aliquot from each tube was streaked onto Skirrow agar plates and incubated in a microaerophilic atmosphere for 48h at 42 ^{o}C. The number of positive enrichements were used to calculate MPN per 100 ml. Confirmation of the

presence of *Campylobacter jejuni* was based on Gram stain, motility, catalase and oxidase tests, antibiotic sensitivity and hippurate test to distiniguish from *Campylobacter coli*.

Secondly, *Clostridium perfringens* ATCC 13124 (Oxoid) was maintained at 4 ^{o}C on RCM broth (boiled to purge of oxygen) and subcultured monthly. Liquid cultures were grown for 24 h in sealed jars at 37 ^{o}C under anoxic conditions. For liquid culture and MPN enumeration, differential reinforced clostridial medium (DRCM; Amyl) was prepared as per manufacturer's instructions. After collection, each sample was heated at 70 ^{o}C for 20 min to select for spore-forming bacteria and serially diluted as per MPN methods described in the Australian standards ^{TM} (AS4276.17.2:2000) All MPN tests were performed in duplicate. Confirmation of the presence of *C. perfringens* was based on sulphite reduction, gelatin liquefaction and acid production. The number of positive enrichments for each dilution were used to calculate the MPN per 100 ml as per Australian standards ^{TM} (AS4276.1:2007).

Pure cultures were enumerated microscopically using a Neubauer haemocytometer and used to prepare standards for real-time PCR. Digester samples with pathogens enumerated by MPN techniques were also used as external standards.

2.5. DNA extraction

DNA extracted from environmental samples often contains high levels of PCR inhibitors such as humic acids. Although DNA extraction kits are faster than conventional DNA extraction methods and provide clean, pure DNA;

yields can be lower. Freeze-thawing improved the yield of DNA from pure cultures of both pathogens and from digester samples (personal observation).

Therefore, total DNA was extracted from freeze-thawed thermophilic digester samples and enumerated bacterial cultures, using a Mo Bio (Solana Beach, CA) UltraClean Soil DNA kit. The procedure was slightly modified from the manufacturer's instructions in the following ways: bead solution was removed and replaced with 0.5 ml buffer equilibrated phenol pH 8.0, chloroform, isoamyl-alcohol (25:24:1) (Sigma-Aldrich, St. Louis, MO); mixed with 0.5 ml digester samples and physically disrupted; the upper layer was then mixed with buffer S1 and applied to the spin column; the amount of buffer S3 was doubled followed by an extra wash of the column with buffer S4. A final elution volume of 30 µl was used to elute purified DNA from the spin column.

2.6. Real-time PCR enumeration

Target specificity is particularly important when measuring pathogens in mixed communities and several previously described real-time PCR primers were tested using DNA purified from the pathogens above and from *Escherichia coli* (WACC 4, PathWest Culture Collection, Perth), *Salmonella typhi* (ATCC 14028), *Enterococcus faecalis* (WACC 28, PathWest, Perth), *Methanoculleus* spp. (bioreactor isolate). Of these, the most promising primer sets were used to quantify different groups (Table 1).

310 Insert Table 1

311

312 Primer pairs selected to quantify all bacteria, *Campylobacter jejuni* or
313 methanogens (Table 1), were included in 20-µl real-time PCR mixtures
314 containing 10 µl SYBR Green mix (IQ SybrGreen Supermix; Bio-Rad), 7 µl
315 distilled H_2O, 1 µl forward primer (10 µM concentration), 1 µl reverse
316 primer (10 µM concentration), and 1 µl DNA. Real-time PCR amplification
317 was conducted on a Rotorgene 3000 (Corbett Life Science, Sydney,
318 Australia) according to the manufacturer's instruction. Amplification was
319 initiated by denaturation at 95°C for 10 min and was followed by up to 40
320 cycles of denaturation at 95°C for 15 s and annealing at 55°C (50 ^{o}C in the
321 case of methanogens) for 30 s, and then by extension at 72°C for 30 s.
322 Fluorescence was acquired during extension using an excitation
323 wavelength of 470 nm and emission detection at 530 nm. A final melting-
324 curve analysis was carried out by continuously monitoring fluorescence
325 between 55°C and 95°C with 0.5°C increments every 10 s. Threshold
326 cycles were calculated automatically by the Rotorgene software (Version
327 6), standardized amounts of DNA extracted from enumerated cultures
328 were included in each run to monitor and correct any between-run
329 variability.
330 Fisher Biotech PCR reagents (Wembley, Western Australia) were used for
331 Taqman real-time PCR for *Clostridium perfringens*. Each 20 µl reaction
332 contained: 1 µl hybridisation probe (2 µM), 2 µl forward primer (10 µM), 2
333 µl reverse primer, 2 µl PCR buffer, 2 µl dNTPs (2mM), 2 µl $MgCl_2$ (25mM),
334 0.2 µl Hotstart Taq, 7.8 µl dH_20 and 1 µl DNA. The cycling conditions were

as follows: an initial 10-min step at 94°C for Taq activation, followed by 35 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 70°C for 10 s. Fluorescence was acquired following the annealing step.

As the levels of PCR inhibitors may vary in each digester sample, it is crucial to estimate PCR efficiency, as this can have a large influence on resulting quantitation according to the equation $N = N_0 \varepsilon^n$ (N_0 is the number of cells/amount of DNA present initially, before the PCR; ε is the efficiency of the PCR; and n is the number of cycles). PCR efficiency (ε) was calculated using the rotorgene software (Version 6). The number of cells in the digester samples (N_0) were calculated from duplicate DNA extractions, calculation of PCR efficiency, and substitution of the constant (N) calculated from the standards into the following equation: $N_0 = N / \varepsilon^n$ (Tichopad *et al*., 2004).

3. Results and discussion

3.1. Chemical properties of raw piggery wastewater and pilot-scale thermophilic digester samples.

Chemical analyses conducted during initial optimisation trials are presented in Table 2. Although the piggery wastewater strength varied seasonally and due to washing procedures, the percentage solids were generally between 0.5 and 3 %; total chemical oxygen demand (TCOD/L) was between 20 and 30 g/L and total ammonia concentration was between 1.8 and 2.4 g/L. Assessment of the initial soluble COD/total COD ratio of the raw piggery wastewater revealed that more than 30% of the initial organic matter had already been solubilized almost entirely to volatile fatty acids by the indigenous microbial populations in the effluent holding sump under ambient conditions. During thermophilic digestion, pH increased from 7.2 to 7.8 and the total COD, soluble COD and total VFAs reduced as they were used up during conversions by the resident microbial communities. Solubilisation of the piggery wastewater was higher in the 55^{o}C reactor (28 % TCOD removed) than the 70 ^{o}C (12% TCOD removed). The predicted biogas production based on mass balance of total COD removed in the 55 ^{o}C thermophilic digester of 49.7 L/day, is higher than the gas production measured (32.4), but could be explained due to the high variability of the influent total COD (SD 9.5 g/L) (Table 2). The predicted biogas production based on total COD removed in the 70 ^{o}C thermophilic digester of 21.7 L/day, is higher than the gas production measured (5.4), but could be explained by the high variability of both the influent and effluent TCOD (9.5 and 5.3 g/L respectively). The methane

374 production rates in the 55 ^{o}C and 70 ^{o}C thermophilic digesters were
375 calculated at 1.02 and 0.12 L/L/day and methane yield 0.14 and 0.04 m^3
376 methane/kg TCOD removed respectively.
377
378 At the higher digestion temperature of 70 ^{o}C, COD reduction was lower and
379 total VFAs remaining were higher; with associated gas production much
380 lower (5 versus 34 L/d at 55 ^{o}C, Table 2) with a lower proportion of methane
381 45 vs 64 %) in the biogas.

382

383 Insert Table 2

384

385 **3.2. Pathogen levels in pilot-scale reactors at different operating**
386 **temperatures.**

387 Samples were collected from pilot-plant thermophilic reactors running at 50,
388 57 and 65v^{o}C and *Clostridium perfringens* and *Campylobacter jejuni* levels
389 measured by MPN techniques. Both pathogens were detected at high levels
390 in raw piggery wastewater (10^5 and 10^3 per gram, respectively). As
391 expected, the number of pathogens surviving thermophilic digestion
392 declined with increasing temperature (Table 3), but some remained, even at
393 a digestion temperature of 65 ^{o}C. The statistical significance was examined
394 using ANOVA and Tukey's pairwise comparison following log transformation
395 of the data to stabilise variance, the levels of *Campylobacter jejuni* were
396 significantly different between 50 and 65^{o}C ($p=0.009$) (Table 3). As high
397 temperatures were not sufficient to remove all pathogens, further samples

398 were collected to investigate other chemical parameters associated with
399 pathogen removal.

400

401 Insert Table 3

402

403 Samples were collected daily from one thermophilic reactor in the pilot plant
404 and pathogens measured by real-time PCR. Viable counts of the pathogens
405 were also measured in the final sample (day 12). Variation in the
406 *Clostridium perfringens* populations, increasing and declining over time, was
407 evident following enumeration by real-time PCR (Fig. 1). This trend was not
408 apparent in the total microbial population, which was monitored to allow for
409 potential differences in consistency between samples. The total microbial
410 population remained relatively constant (between 6 and 6.7 x 10^9 per ml, SD
411 0.2), so the observed variation was unlikely to reflect sample inconsistency.
412 Low levels (20 to 80 per 100 ml) of *Campylobacter jejuni* were detected by
413 culturing and real-time PCR in the raw effluent sample and reactor samples
414 on day 2 and 12 where gas production rate was lowest (1.8 L/h).

415 Gas production was measured at hourly intervals and averaged over the 24
416 hour period immediately preceding sample collection and plotted alongside
417 the calculated *Clostridium perfringens* populations (Fig. 1). There was a
418 striking inversely proportional relationship between gas production and
419 number of *Clostridium perfringens*. Regression analysis of gas production
420 and number of *Clostridium perfringens* showed a significant correlation

421 (p=0.0017, r^2 0.81). As Clostridia produce hydrogen during anaerobic
422 breakdown of organic waste, a positive correlation might be expected. If
423 *Clostridium perfringens* cells were competing with other Clostridia for
424 substrates, the observed negative correlation may be the result.

425

426 Insert Figure 1

427

428 To investigate this correlation further, a second set of samples was
429 collected weekly, both before and after effluent addition, to one thermophilic
430 digester over a longer four weekly period (Fig. 2). The reactor was running
431 at a higher temperature of 65 ^{o}C. Gas production declined over the course
432 of the experiment from 0.8 to 0.47 L/h by the end, perhaps reflecting
433 declining substrate availability. Levels of both bacterial pathogens were high
434 at the start of the trial (Fig. 2a and 2b). This may have resulted from higher
435 initial substrate levels. Following the high reading in the first samples,
436 *Clostridium perfringens* levels peaked when gas production was lowest (Fig.
437 2a), supporting previous findings, although the correlation using regression
438 analysis was not significant. Despite the low number of samples,
439 *Campylobacter jejuni* levels were significantly higher where gas production
440 was lowest (Fig. 2b) (p=0.057, r^2 0.89).

441

442 Insert Figure 2

443

444 Levels of both pathogens were slightly lower during this trial, likely to reflect
445 the higher operating temperature (65 vs 58 ^{o}C). However, at both
446 temperatures, gas production appeared to be inversely proportional to
447 pathogen number. Gas production also varied between the two pilot-scale
448 thermophilic digesters which were set up in parallel, probably as a result of
449 the differences in resident bacterial communities.

450 Viable counts and real-time PCR of *Clostridium perfringens* were
451 determined in both thermophilic digesters during a third sampling period
452 with digesters running at 50 ^{o}C over a three-month period. On one
453 occasion, the levels of pathogens in the two reactors varied considerably
454 (June 2007), despite running at the same temperature using the same raw
455 effluent (Table 4). Reactor two had a mean gas production more than
456 double that of thermophilic reactor one (1.13 vs 0.45 litres per hour). This
457 correlated with considerably lower numbers of pathogens surviving (Table
458 4). Regression analysis showed that there was a significant correlation
459 between gas production and level of *Clostridium perfringens* with a p-value
460 of 0.007 and r^2 0.86 (Fig. 3).

461

462 Insert Table 4 and Figure 3

463

3.3. Methanogen levels in pilot-scale reactors at different operating temperatures.

The terminal and rate-limiting step of anaerobic digestion is methanogenesis, with methanogens converting hydrogen and carbon dioxide to methane. It would be expected that the number or acitivity of methanogens would be higher in samples with higher levels of gas production and therefore, methanogens may also be an important indicator of pathogen removal. Only trace levels of hydrogen were present in the thermophilic reactors running at 55^{o}C with the gas consisting partly CO_2 (30-40%) and the remainder methane (Table 2).

The numbers of methanogens in samples with high pathogen numbers and low gas production (* Fig. 1 and 2) and low pathogen numbers and high gas production (# Fig. 1 and 2) were compared. The microbial population was almost twice as high when gas production was high (4.3 x 10^{10} SE 0.7 vs. 2.5 x 10^{10} SE 0.4), with methanogen levels almost three-times as high (5.50 x 10^{8} SE 1.2 vs. 1.90 x 10^{8} SE 0.6). Although piggery wastewater contained predominantly hydrogen-utilising methanogens, aceticlastic methanogens related to *Methanosarcina thermophila* appeared to rapidly predominate in the reactors during anaerobic digestion at the relatively high pH found in piggery wastewater(data not shown).

3.4. Pathogen levels in laboratory scale reactors using different strength piggery wastewater.

The correlation between gas production and pathogen levels was investigated further in continuously stirred bench scale reactors (CSTR) which allowed closer control of temperature and monitoring of chemical properties. The lab-scale reactors were run at a short 2 d HRT to investigate solubilisation, rather than maximise methane production. The first trial investigated the effect of feed strength (low, medium and high) on methane production, pathogen levels and chemical properties. With less available substrates, methane production would be expected to be lower and this may influence pathogen removal. Loading rates were comparable between the bench scale reactors (2.2 low, 3.5 mid and 6.4 high gTCOD L^{-1} d^{-1}) and pilot-scale digesters (3.7 gTCOD L^{-1} d^{-1}). Maximum TCOD removal was achieved using mid-strength feed (29%), with high strength and low strength feed lower (19 and 1 % respectively). Methane yield was almost twice as high in the mid-strength compared to the high strength (0.27 vs 0.15). The methane yield in the low strength appeared to be much higher (1.9), but this was likely a result of inaccuracy of measuring very low amounts of TCOD removal and large standard deviation. Methane yield was similar between the pilot-scale reactors operating at 55 ^{o}C and bench-scale reactors using full-strength feed water as feed, despite the different HRT of 7d and 2d HRT respectively.

Mid strength feed produced a higher methane yield (0.27) in the bench-scale reactors that the pilot-scale reactors, likely due to dilution of inhibitors present in high strength feed (Table 5) and improved mixing. In another study with fixed-bed reactors treating piggery wastewater, Sanchez et al.

511 (2006) also found optimal removal efficiency at 4 to 8 g TCOD L^{-1}, with
512 decreasing efficiency at higher concentrations.

513 The highest level of methanogens (8 x 10^7) was found using the mid
514 strength piggery wastewater feed, which also showed the highest
515 pathogen kill (30%) (Table 5). Two-way ANOVA showed the methane yield
516 from all three feed strengths were significantly different ($p<0.001$).
517 However, there was no significant difference between methanogen or
518 *Clostridium perfringens* levels, largely due to variations between
519 replicates. The number of methanogens was >10 fold higher in the
520 benchscale thermophilic reactors than in the pilot-scale thermophilic
521 reactors, irrespective of feed strength, which could reflect better mixing
522 and substrate availability.
523

524 Insert Table 5.

525

526 In other studies (L. Ho 2008, unpublished), artificially lowering the pH of
527 piggery wastewater from 8.2 to 5.5 inhibited methanogenesis but enhanced
528 solubilisation and acidification. By contrast, lowering the pH to 6.5 enhanced
529 substrate utilisation and methane production (data not shown). The effect of
530 lowering pH on pathogen survival was therefore investigated (Figure 4).
531 Levels of *Clostridium perfringens* and methanogens were measured by real-
532 time PCR in vials after 10 days incubation. Methane production rate
533 increased as pH was lowered, with cumulative methane production 33 ml at

534 pH 6.5, 22.5 at pH 7, 16 ml at pH 7.5 and 7 ml at pH 8.3. Methanogen
535 numbers were correspondingly higher (1 x 10^4 at pH 8.2 and 8 x 10^5 at pH
536 6.5) and *Clostridium perfringens* were lower (2.5 x 10^4 at pH 8.2 and 2 x 10^3
537 at pH 6.5) (Figure 4).

538

539 Insert Figure 4

540

541 Under conditions which increased gas production such as increasing feed
542 strength or decreasing pH from 8.2 to 6.5, *Clostridium perfringens* survival
543 was correspondingly lower.

Discussion

Optimal thermophilic digestion temperature in anaerobic digesters is often a compromise between the best temperature for solubilisation and the best for pathogen destruction. Both the pilot-plant thermophilic digesters and bench-scale digesters were operated in the same way by draw and fill. They also used the same wastewater as influent. The data presented here from each reactor differ in hydraulic residence time (HRT) and feed strength. Initially both systems were operated on a 4 d HRT but 7 d was selected for the pilot scale system as optimal for digestion of solids, to produce nutrients and increase the availability of inorganic substrates. The lab-scale reactors were run at a short, 2d HRT, to encourage acidification, although this was achieved with synthetic wastewater in prior studies, acidification was not achieved with piggery wastewater due to its large buffering capacity. Other studies have shown up to 78% removal of TCOD in some similar systems (Sakar et al. 2009). The 55 ^{o}C pilot-scale thermophilic reactor was operating at 28% TCOD removal with a methane yield of 0.14 m^3/kg TCOD removed. The TCOD removal and methane yield were lower when operated at 70^{o}C (12.4% and 0.04 respectively). The TCOD removal and methane yield were slightly higher in the bench-scale reactors (29% and 0.27 respectively with the mid-strength feed), likely to result from better mixing, despite the sub-optimal 2d HRT.

Although increasing temperature and retention time improves pathogen kill, in this system, optimal solubilisation was achieved at 55 ^{o}C. At a temperature of 65 ^{o}C, quite high levels of viable *Clostridium perfringens* remained (424 per 100 ml) and some *Campylobacter jejuni* survived (5 per

100 ml). Even at temperatures above 65 ^{o}C, some viable *Clostridium perfringens* and *Campylobacter jejuni* remained. This may have resulted from insufficient mixing in the pilot-scale digesters (130 L), reducing the effective hydraulic residence time (HRT). Experiments were therefore also carried out in bench-scale reactors and batch vials, with better mixing and less heterogeneity.

Although pathogen survival in thermophilic digestion is undoubtedly temperature related (Bendixen, 1994), in both pilot and laboratory scale reactors, increased gas production in addition to temperature, appeared to correlate with lower levels of *Clostridium perfringens* and *Campylobacter jejuni*. The correlation was less pronounced with *Campylobacter jejuni* which may have reflected their lower density (6550 vs >11 000 per 100 ml) or different mechanisms of heat resistance. On some occasions, *C. jejuni* levels were below the limit of detection by real-time PCR (approximately 10 cells/ml) and could not be detected reliably in samples from bench-scale reactors. *C. jejuni* does not sporulate and reportedly loses viability rapidly at temperatures above 50 °C (ICMSF, 1996), however above 56 ^{o}C the cells demonstrated a non-logarithmic reduction in numbers, resulting in a tailing effect which could be significant. It has also been shown that a stress response can be induced in *C. jejuni* at the alkaline pH found in piggery wastewater (Wu *et al*., 1994). Other species of bacterial pathogens were investigated but appeared to be removed effectively by thermophilic digestion at these temperatures, so it is not clear whether the correlation between methane production and pathogen kill is confined to *Clostridium perfringens* and *Campylobacter jejuni*.

Clostridium perfringens, as a spore-forming hydrogen producer, might be expected to increase in number as methane production increases, with the hydrogen produced converted to methane by hydrogenotrophic methanogenic groups, but the opposite trend was observed. The observed negative correlation may reflect inhibition of hydrogen-consuming groups and/or competition with other spore-formers for limited substrates. For example, the growth of homoacetogenic Clostridia (eg. *Clostridium thermoaceticum*) could outcompete *Clostridium perfringens* for available substrates, increasing the amount of acetate produced with more available for conversion to methane by the aceticlastic methanogens. This explanation would be supported by the observation that aceticlastic groups of methanogens such as *Methanosarcina thermophila* appeared to predominate over hydrogen-utilising groups in the thermophilic reactors (personal observation). The growth of the pathogens may also be inhibited by indigenous microorganisms (Sidhu et al., 2001) or reflect reduced activity (and hydrogen production) during conditions which favour sporulation. Although neither method of bacterial quantitation, real-time PCR or MPN, could distinguish between spores and vegetative cells, it would be of interest to know the proportion of each.

The growth or activity of bacteria in the digester communities are likely to influence pathogen survival through competition for available substrates or the production of inhibitory compounds. In the case of *Clostridium perfringens*, sporulation and subsequent survival has been shown to be influenced by the presence of *Bacteroides fragilis* and short-chain fatty acids (Wrigley 2004). Under conditions which favour hydrogen producing spore-

formers, such as increasing digestion temperatures, hydrogen-consuming groups may decline, with more hydrogen available for methanogenesis. An increase in number and proportion of hydrogen-consuming methanogens has been reported during thermophilic digestion at 65^{o}C compared to 55^{o}C (Ahring et al., 2001). We also found that increased gas production correlated with higher levels of methanogens. However, a number of other factors are known to influence methane production. High concentrations of ammonia are inhibitory, particularly to hydrogen-consuming methanogens. The total ammonia concentration in the undiluted piggery wastewater used in both pilot and laboratory scale reactor experiments were between 1.8 and 2.4 g/L which is reportedly inhibitory to methanogens at pH above 7.6 (Hashimoto, 1986). Artificially lowering the pH in bench-scale experiments, increased methane production and reduced pathogen survival, and coincided with the lowest levels of free ammonia.

A correlation between the survival of these two pathogens has been previously reported (Skanseng et al, 2006), although it is not clear whether this occurred because of direct interactions or indirectly with similar conditions regulating their heat resistance mechanisms. Work is continuing on comparing the bacterial and archaeal communities in these digester samples to examine changes which may be associated with increased methane production and improved pathogen removal.

642 **4. Conclusions**

643 ● During thermophilic anaerobic digestion, to capture energy and nutrients
644 from piggery effluent, higher temperatures reduced the survival of the
645 bacterial pathogens *Campylobacter jejuni* and *Clostridium perfringens* as
646 expected. However, higher levels of gas production also correlated with
647 lower pathogen levels, irrespective of temperature.

648 ● This correlation was confirmed under different operating temperatures
649 over periods of a week, several weeks and months. It did not appear to
650 result from variation in the size of the microbial digestion communities
651 although the types of bacteria will undoubtedly influence digestion and
652 subsequent hydrogen production.

653 ● The terminal step in anaerobic digestion is the conversion of hydrogen
654 and carbon dioxide and acetate to methane by methanogens. Methane can
655 be captured and used as an energy source. The number of methanogens in
656 reactor samples correlated with increased methane production and
657 enhanced pathogen kill.

658 ● Under conditions which enhanced gas production in lab-scale reactors,
659 such as increased organic loading rate or reduced pH (6.5), pathogen
660 removal was also enhanced.

661

662

663

Acknowledgements

This work was supported by the Australian Government through the Environmental Biotechnology Cooperative Research Centre (EBCRC) and the South Australian Research and Development Insititute (SARDI). Assistance from the team at Roseworthy including Paul Harris, Sandy Wyatt and Belinda Rodda is gratefully acknowledged.

References

Ahring, B.K., Ibrahim, A.A. and Mladenovska, Z. (2001) Effect of temperature increase from 55 °C to 65 °C on performance and microbial population dynamics of an anaerobic reactor treating cattle manure. Water Research 35, 2446-2452.

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A. and Raskin, L. (1996) The oligonucleotide probe database. Applied and Environmental Microbiology 62, 3557-3559.

American Public Health Association, American Water Works Association, Water Pollution Control Federation 1989. *Standard* Methods for the Examination of Water and Wastewater, 17th edition. Washington DC, USA 40

Angelidaki,I., Ellegaard,L. and Ahring,B.K. (1999) A comprehensive model of anaerobic bioconversion of complex substrates to biogas. Biotechnology and Bioengineering 63, 363-372.

Batstone,D.J., Keller,J., Angelidaki,I., Kalyuzhny,S.V., Pavlostathis,S.G., Rozzi,A., Sanders,W.T.M., Siegrist,H. and Vavilin,V.A. (2002) Anaerobic digestion model No. 1 (ADM1). IWA Publishing, London, UK. (Scientific and Technical Report 13).

Bendixen, H.J. (1994). Safeguards against pathogens in Danish biogas plants. Water Science and Technology 30,171-180.

Bolton, F. J. and Robertson, L. (1982) A selective medium for isolation of *Campylobacter jejuni/coli.* Journal of Clinical Pathology 35, 462-467.

Boopathy, R. (1998) Biological treatment of swine waste using anaerobic baffled reactors. Bioresource Technology 64, 1–6.

Burtscher, C., Fall, P.A., Christ, O., Wilderer, P.A. and Wuertz, S. (1998) Detection and survival of pathogens during two stage thermophilic/mesophilic anaerobic treatment of suspended organic waste. Water Science and Technology 38,123-126.

Chinivasagam, H. N., Thomas, R.J. Casey, K., McGaham, E., Gardner, E.A. and Rafiee, M. (2004) Microbiological status of piggery wastewaterfrom 13 piggeries in the south east Queensland region of Australia, Journal of Applied Microbiology 97, 883-891.

Easterbrook, T.J. and West P.A. (1987) Comparison of most probable number and pour plate procedures for isolation and enumeration of sulphite-reducing Clostridium spores and group D faecal streptococci from oysters. Journal of Applied Bacteriology 62, 413-9.

Fukushima, H., Tsunomori, Y. and Seki, R. (2003) Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools. Journal of Clinical Microbiology 41, 5134-46.

George, H. A., Hoffman, P.S., Smibert, S.M. and Krieg, N.R. (1978) Improved media for growth and aerotolerance of *Campylobacter fetus.* Journal of Clinical Microbiology 8, 36-41.

Guerrero L., Omil F., Mendez R. and Lema J.M. (1999). Anaerobic hydrolysis and acidogenesis of wastewaters of food industries with high content of organic solids and protein. Water Research 33, 3281-90.

Hansen, K.H., Angelidaki, I. and Ahring, B.K. (1998) Anaerobic digestion of swine manure: inhibition by ammonia. Water Research 32, 5–12.

Hashimoto, A.G. (1986) Ammonia inhibition of methanogenesis from cattle wastes. Agricultural Wastes 17, 241-261.

Hill, V.R. (2003) Prospects for pathogen reductions in livestock wastewaters: A review. Critical Reviews in Environmental Science and Technology 33, 187-235.

Horan, N.J., Fletcher, L. Betmal, S.M., Wilks, S.A. and Keevil, C.W. (2004) Die-off of enteric bacterial pathogens during mesophilic anaerobic digestion. Water Research 38,1113-1120.

ICMSF (International Commission on Microbiological Specifications for Foods). (1996) Micro-organisms in foods 5. Microbiological specifications of food pathogens. Blackie Academic and Professional, London.

Kaparaju, P. and Rintala, J. (2005) Anaerobic co-digestion of potato tuber and its industrial by-products with pig manure. Resources, Conservation and Recycling 43, 175–188.

Kunte, D.P.M., Yeole, T.Y., Chiplonkar, S.A. and Ranade, D.R. (1998). Inactivation of *Salmonella typhi* by high levels of volatile fatty acids during anaerobic digestion, Journal of Applied Microbiology 84, 138-142.

Lettinga, G. and Hulshoff Pol, L.W. (1991) UASB-process Design for Various Types of Wastewaters. Water Science and Technology 24, 87-107.

Lo, K.V., Liao, P.H. and Gao, Y.C. (1994) Anaerobic treatment of swine wastewater using hybrid UASB reactors. Bioresource Technology. 47, 153–157.

Murphy, C., Carroll, C. and Jordan, K.N. (2005) Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. Journal of Applied Microbiology 100, 623-632.

Nielsen, H.B. and Ahring, B.K. (2007) Effect of tryptone and ammonia on the biogas process in continuously stirred tank reactors treating cattle manure. Environmental Technology 28**,** 905-914**.**

Olsen, J. E. and Larsen, H.E. (1987) Bacterial decimation times in anaerobic digestions of animals slurries. Biological Wastes 21, 153-168.

Sahlstrom, L., (2003), A review of survival of pathogenic bacteria in organic waste used in biogas plants, Bioresource Technology 87, 161-166.

Sakar, S., Yetilmezsoy, K. and Kocak, E. (2009) Anaerobic digestion technology in poultry and livestock waste treatment: a literature review. Waste Management Research 27; 3.

Salsali, H., Parker, W.J., and Sattar, S.A. (2008) The effect of volatile fatty acids on the inactivation of *Clostridium perfringens* in anaerobic digestion. World Journal of Molecular Biotechnology 24, 659-665.

Sanchez, E., Borja, R., Travieso, L., Martin, A. and Colmenarejo, M.F. (2005) Effect of organic loading rate on the stability, operational parameters and performance of a secondary upflow anaerobic sludge bed reactor treating piggery waste. Bioresource Technology 96, 335-344.

Sanchez, E., Borja, R., Travieso, L., Martin, A., Colmenarejo, M.F. and Nikolaeva, S. (2006) An evaluation of variables affecting the stability and performance of down-flow anaerobic fixed bed reactors treating piggery *Environmental Technology, 27: 85-93.*

Sidhu, J., Gibbs, R.A., Ho, G.E. and Unkovich, I. (2001) *Selection of Salmonella typhimurium* as an indicator for pathogen regrowth potential in composted biosolids Letters in Applied Microbiology 29, 303-307.

Skanseng, B., Kaldhusdal M., and Rudi, K. (2006) Comparison of chicken gut colonisation by the pathogens *Campylobacter jejuni* and *Clostridium perfringens* by real-time quantitative PCR. Molecular and Cellular Probes 20, 269-279.

Skirrow, M.B. (1977) *Campylobacter enteritidis*: a "new" disease. British Medical Journal 2, 9-11.

Smith, S.R., Lang, N.L., Cheung, K.M. and Spanoudaki, K. (2005) Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Management 25, 417-425.

Stams, A.J.M. (2003) Metabolic interactions between methanogenic consortia and anaerobic respiring bacteria. Antonie Van Leeuwenhoek 66, 271-294.

Stintzi, A. (2003) Gene expression profile of *Campylobacter jejuni* in response to growth temperature variation. Journal of Bacteriology 185, 2009-2016.

Tichopad A., Dilger, M., Schwarz, G. and Pfaffl, M.W. (2003) Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Research 31, 122.

Vanotti, M.B., Millner, P.D., Hunt, P.G. and Ellison, E.Q. (2005) Removal of pathogen and indicator microorganisms from liquid swine manure in multi-step biological and chemical treatment. Bioresource Technology 96, 209-214.

Wilson, C.A., Murthy, S.M. and Fang, Y. (2008) The effect of temperature on the performance and stability of thermophilic anaerobic digestion. Water Science and Technology 57, 297-304.

Wise, M.G. and Siragosa, G.R. (2005) Quantitative detection of *Clostridium perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. Applied and Environmental Microbiology 71, 3911-16.

Wrigley, D. M., (2004) Inhibition of *Clostridium perfringens* sporulation by *Bacteroides fragilis* and short-chain fatty acids. Anaerobe 10, 295-300.

Wu, C., Clos, J., Giorgi, G., Haroun, R.I., Kim, S-J., Rabindran, S.K., Westwood, J.T., Wisniewski, J. and Yim, G. (1994) Structure and regulation of heat shock transcription factor. In The biology of heat shock proteins and molecular chaperones (ed. R.I. Morimoto, A. Tissieres, C. Georgopoulos), 395-416. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Yu H.Q. and Fang H.H.P. (2001) Acidification of lactose in wastewater. Journal of Environmental Engineering127, 825-831.

Zhang, R.H., Yin, Y., Sung, S. and Dague, R.R. (1997) Anaerobic treatment

of swine waste by the anaerobic sequencing batch reactor. *Transactions of the ASAE* 40, 761–767.

Zhang, R.H., Tao, J. and Dugba, P.N. (2000) Evaluation of two-stage anaerobic sequencing batch reactor systems for animal wastewater treatment. Trans American Society of Agricultural Engineering 43, 1795–1801.

Primer	Target Group	Position on 16S	Sequence †	. Reference
Fq	16S Gene All bacteria and methanogens	1097	CGGCAACGAGCGCAACCC	Christophersen *et al*. pers.comm.
Rq	16S Gene All bacteria and methanogens	1221	CCATTGTAGCACGTGTGTAGCC	Christophersen *et al*. pers.comm.
MET630F	16S Gene Methanogens	630	GGATTAGATACCCSGGTAGT	Christophersen *et al*. pers.comm.
MET803R	16S Gene Methanogens	803	GTTGARTCCAATTAAACCGCA	Christophersen *et al*. pers.comm.
CampF	DNA gyrase (GyrA)	NA	TGGGTGCTGTTATAGGTCGT	Fukushima *et al.* 2003
CampR	DNA gyrase (GyrA)	NA	GCTCATGAGAAAGTTTACTC	Fukushima *et al.* 2003
CperfF	16S Gene *Clostridium perfringens*	176	CGCATAACGTTGAAAGATGG	Wise and Siragusa, 2005
CperfR	16S Gene *Clostridium perfringens*	258	CCTTGGTAGGCCGTTACCC	Wise and Siragusa, 2005
Cperf probe		190	5'-[FAM]TCATCATTCAACC AAAGGAGCAATCC[TAMRA]3'	Wise and Siragusa, 2005

Table 1 - PCR primers selected for quantitation of microbial populations in thermophilic digester samples. Presented according to the oligonucleotide probe nomenclature (Alm *et al*., 1996).

Parameter	Unit	Piggery Wastewater (Influent)	Thermophilic Digester (Effluent)	Thermophilic Digester (Effluent)
Temperature			55	70
HRT	days		7	7
pH		7.2 (0.2)	7.8	7.8
Loading Rate	g/L/day		3.73 (0.9)	3.73 (0.9)
Total chemical oxygen demand	g TCOD/L	25.0 (9.5)	17.9 (1.5)	21.9 (5.3)
Soluble chemical oxygen demand	g SCOD/L	10.3 (0.5)	5.7 (1.2)	9.5 (2.6)
Total volatile fatty acids	g VFA COD/L	1.65 (0.01)	0.46 (0.12)	0.67 (0.5)
Acetate	mg/L	736 (118)	233 (75)	278 (185)
Propionate	mg/L	250 (18)	114 (16)	131 (103)
Ammonium-nitrogen	mg /L	1361 (319)	1728 (596)	1611 (346)
Gas Production	L/d		32.4 (15.4)	5.4 (3.8)
Carbon Dioxide	%		35	50
Methane	%		63.5	45
Methane production rate	L/L/d		1.02 (0.49)	0.12 (0.09)
Methane yield	m^3/kg TCOD removed		0.15 (8 %)	0.04 (24 %)

Table 2 - Chemical analysis of piggery wastewater and pilot plant thermophilic digesters running at 55 and 70 ^{o}C, over a 3 week period, standard deviation indicated.

Digestion Temperature	*Clostridium perfringens* per 100ml	*Campylobacter jejuni* per 100ml
Raw Effluent	>11 000	~1000
65°C, 7d HRT	424 (94)	5 (3)[a]
57°C, 7d HRT	615 (248)	50 (26)[ab]
50°C, 7d HRT	1670 (719)	393 (207)[b]

Table 3 - Levels of pathogens measured by MPN culture methods in pilot-scale thermophilic reactors at different temperatures, 7d HRT. Standard errors indicated, numbers with different superscripts are significantly different and p=0.05.

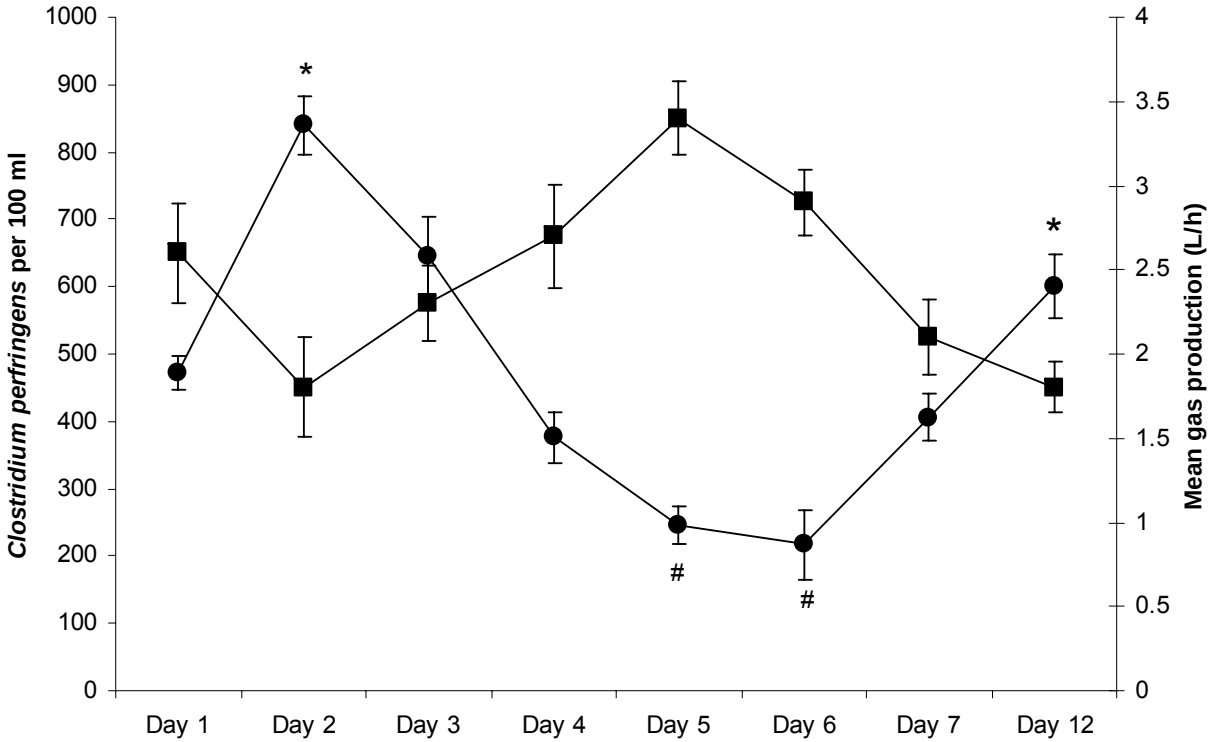
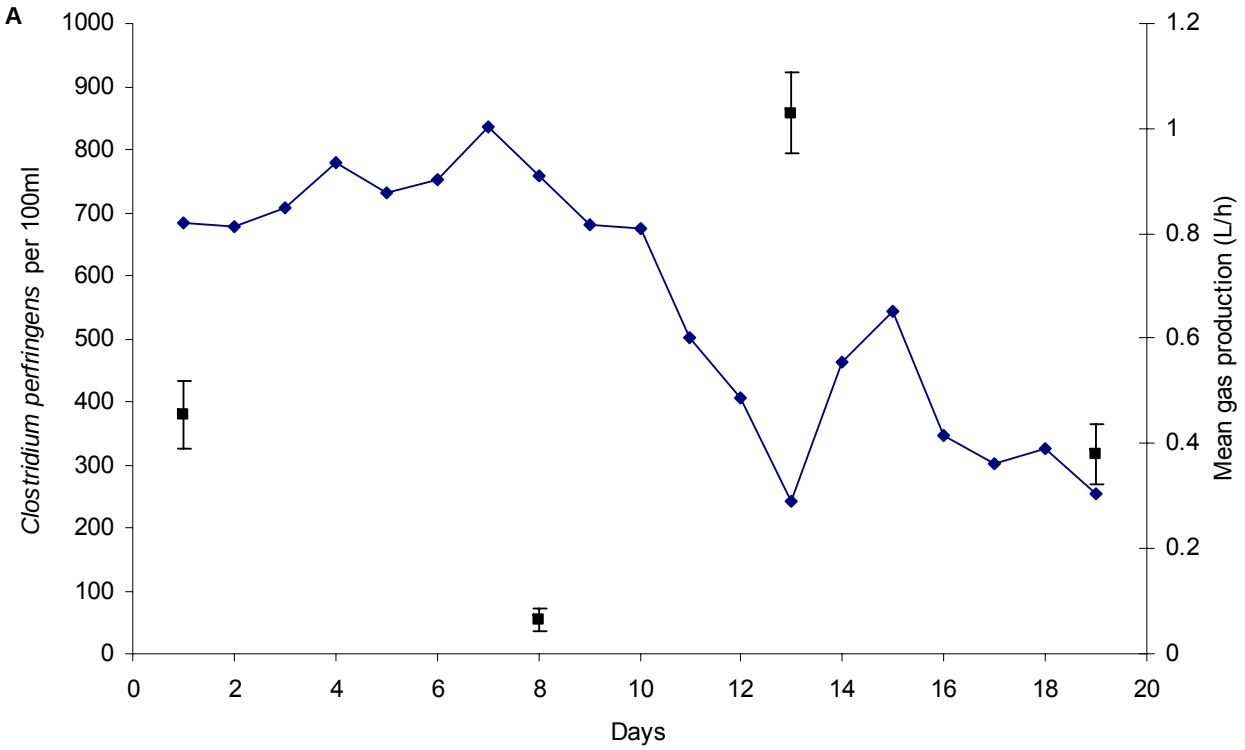


Fig. 1 - Levels of *Clostridium perfringens* (•) in samples collected daily from a pilot scale thermophilic reactors (57 ^{o}C, 7d HRT), measured by real-time PCR and corrected to total microbial population. Mean gas production in the 24 h period preceding sampling is presented on the secondary axis (■). Standard errors are indicated. Methanogen levels were compared in samples marked # and *.


A
1000
900
800
700
600
500
400
300
200
100
0
Clostridium perfringens per 100ml
1.2
1
0.8
0.6
0.4
0.2
0
Mean gas production (L/h)
0
2
4
6
8
10
12
14
16
18
20
Days

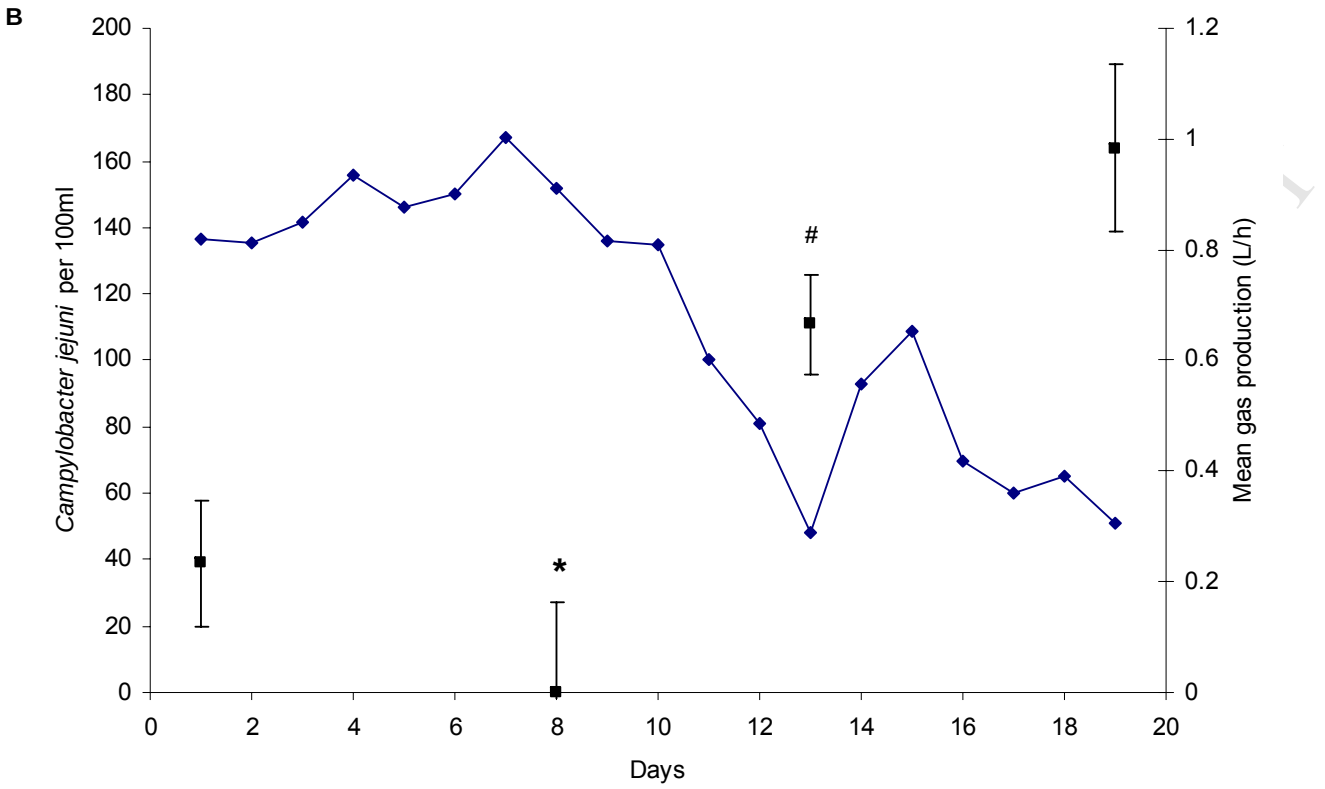


Fig. 2 - Gas production (mean L/h) (SE between 0.03 and 0.05) and pathogen counts measured by real-time PCR during a four week trial with samples collected on day 7, 14, 21 and 28. Thermophilic digesters were running at 65 ^{o}C (+/- 2), 7d HRT. Pathogen counts, corrected to the total microbial population, are presented on secondary axes in graph A (*Clostridium perfringens*) and graph B (*Campylobacter jejuni*) with standard errors indicated. Methanogen levels were compared in samples marked # and *.

Sample	*Gas production (mean L/h)*	*Clostridium perfringens* (MPN) per 100 ml	*Clostridium perfringens* (real-time PCR) per 100 ml	*Campylobacter jejuni* (real-time PCR) per 100 ml
Raw Effluent	N/A	11,000	>11,000	~5000
T1 April 2007	1	ND	233	0.65
T2 April 2007	0.64	ND	501	0.9
T1 June 2007	0.45	3,100	813	780
T2 June 2007	1.13	240	364	6.8
T1 Nov 2007	0.39	11,000	10000	15
T2 Nov 2007	0.28	7800	9600	23.4

Table 4 - Viable counts and real-time PCR of pathogens in samples collected from both thermophilic reactors running at 50 ^{o}C, 7d HRT.

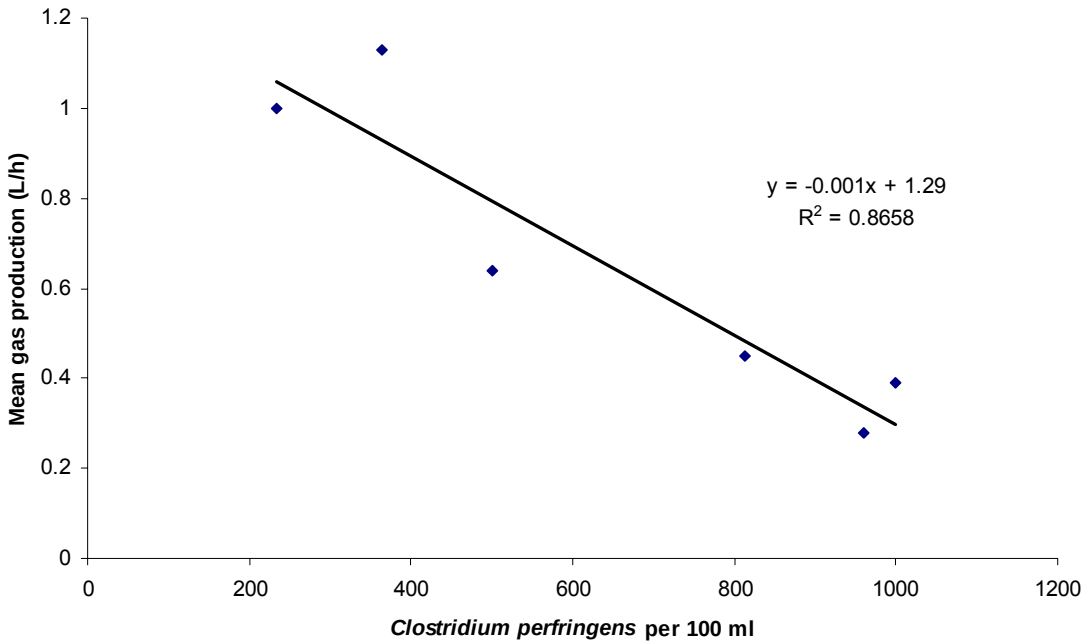


Fig. 3 - Correlation (p-value 0.007) between gas production and *Clostridium perfringens* populations measured by real-time PCR in two parallel pilot-scale thermophilic anaerobic digesters running at 50 ^{o}C and 7d HRT.

Parameter	Unit	Low strength	Mid Strength	High Strength
Temperature		55	55	55
HRT	days	2	2	2
Loading Rate	gTCOD/L/d	2.2	3.5	6.4
Influent TCOD	g TCOD/L	4.44 (0.40)	7.12 (0.49)	12.87 (0.77)
Influent SCOD	g SCOD/L	2.70 (0.12)	5.18 (0.29)	7.62 (0.75)
Total Alkalinity	g $CaCO_3$	2.19 (0.17)	3.60 (0)	5.60 (0.49)
Effluent TCOD	g TCOD/L	4.38 (0.40)	5.05 (0.45)	10.37 (0.77)
Effluent SCOD	g SCOD/L	2.75 (0.12)	3.65 (0.23)	7.12 (0.42)
Total Alkalinity	g $CaCO_3$	2.51 (0.07)	4.01 (0.12)	6.50 (0.17)
Total volatile fatty acids	g VFA-COD/L	2.22 (0.24)	2.55 (0.19)	6.01 (0.31)
Acetate (C2)	mg COD/L	761 (97)	1003 (113)	2652 (141)
Propionate (C3)	mg COD/L	474 (59)	1074 (91)	1964 (169)
Ammonium-nitrogen	mg /L	690 (61)	968 (28)	2150 (191)
Carbon Dioxide	%	24 (2)	20 (1)	30 (2)
Methane	%	65 (3)	84 (2)	72 (1)
Biogas Prod. rate	L/L/d	0.093 (0.01)	0.329 (0.016)	0.249 (0.019)
Methane Prod. rate	L/L/d	0.06 (0.006)	0.28 (0.013)	0.19 (0.015)
Methane yield	m^3/kg TCOD removed	1.9 (9 %)[# a]	0.27 (9 %)[b]	0.15 (6%)[c]
Methanogens per ml	x 10^7	4.65 (0.4)	8.17 (0.9)	7.7 (0.8)

Clostridium perfringens per 100 ml	229 (180 in)	279 (400 in)	350 (425 in)
% reduction	N/A	30%	18%

Table 5 - Chemical composition and analyses of lab-scale thermophilic digesters, running at 55 ^{o}C, 2d HRT with three feed strengths. Standard deviations indicated, methane yields with different superscripts are significantly different (p=0.001).$^{\#}$ Methane yield is likely to reflect inaccuracy in measuring the low TCOD removal using the low-strength feed.

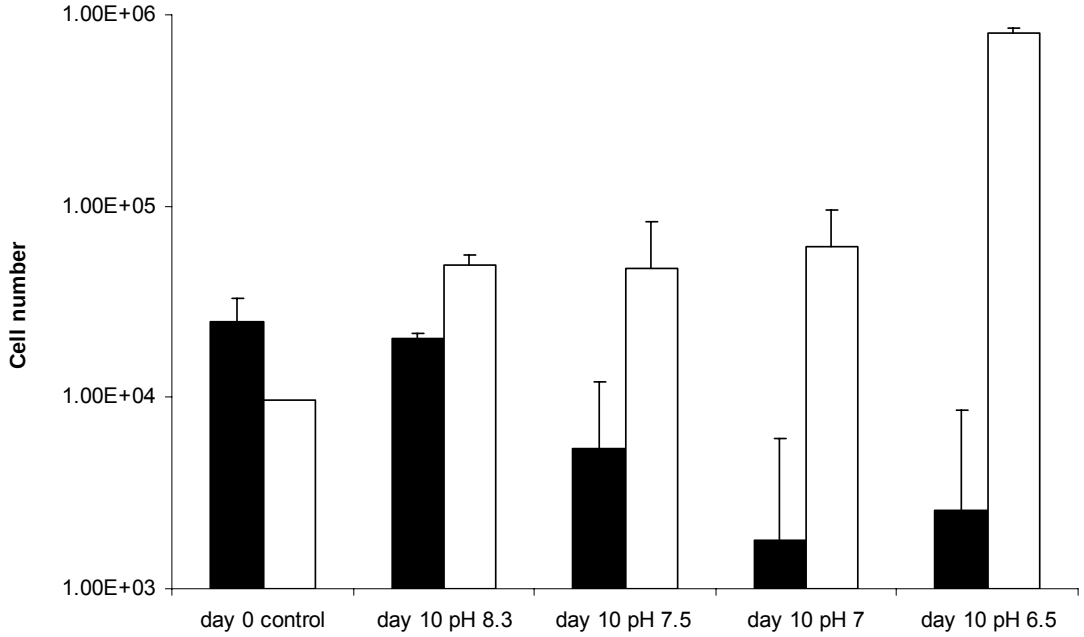


Figure 4: Methanogens per ml (□) and *Clostridium perfringens* per 100 ml (■) levels in samples collected from batch vial experiments with pH levels between 6.5 and 8.3, measured by real-time PCR and corrected to total microbial population. SE indicated.